

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Sherman FONG, *et al.*

Application Serial No. 10/791,618

Filed: March 2, 2004

For: **NOVEL POLYPEPTIDES AND  
NUCLEIC ACIDS ENCODING  
BOLEKINE**

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) Examiner: DeBerry, Regina M.  
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) Art Unit: 1647  
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) Confirmation No: 4005  
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) Attorney's Docket No. 39780-1192-2C1  
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**ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES**

**APPELLANTS' BRIEF**

**MAIL STOP APPEAL BRIEF - PATENTS**

Commissioner for Patents -

P.O. Box 1450

Alexandria, Virginia 22313-1450

Dear Sir:

On January 16, 2007, the Examiner made a final rejection to pending Claims 12-14. A Notice of Appeal was filed on April 12, 2007.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. A request for a 5 month extension of time is filed concurrently herewith.

The following constitutes Appellants' Brief on Appeal.

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**1. REAL PARTY IN INTEREST**

The real party in interest is Genentech, Inc., South San Francisco, California, by an assignment of the parent application U.S. Serial No. 09/816,920, recorded 07/05/2001, at Reel 011717 and Frame 0254.

**2. RELATED APPEALS AND INTERFERENCES**

There are no related appeals or interferences.

**3. STATUS OF CLAIMS**

Claims 1-18 are in this application.

Claims 1-11 and 15-18 are canceled.

Claims 12-14 stand rejected and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided as Appendix A.

**4. STATUS OF AMENDMENTS**

All amendments, including the cancellation of non-elected claims 1-11 and 15-18, have been entered.

**5. SUMMARY OF CLAIMED SUBJECT MATTER**

Claim 12 is drawn to a method enhancing the infiltration of immune cells in a mammal, comprising administering to the mammal an effective amount of Bolekine polypeptide as shown in Figure 2 (SEQ ID NO: 2). Bolekine is defined on page 7, lines 13-34 of the specification, and its amino acid sequence is shown in Figure 2, SEQ ID NO: 2. The preparation of Bolekine is described on page 56, line 36 – page 62, line 22, and in Examples 1, 3, 4 and 5. Example 10 describes experimental results showing that Bolekine exhibited T lymphocyte proliferation stimulating activity in the Mixed Lymphocyte Reaction (MLR) assay. Example 11 describes experimental data showing that Bolekine stimulates an immune response by inducing immune cell infiltration in the Vascular Permeability Assay.

Dependent claim 13 recites that the immune cells are mononuclear cells, eosinophils, or polymorphonuclear eutrophils (PMNs). Support for this claim is at least in Example 11, lines 3 and 11-12.

Independent claim 14 is drawn to a method of alleviating infection in a mammal comprising administering an effective amount of Bolekine as shown in Figure 2 (SEQ ID NO: 2). Specific support for this claim is in Examples 10 and 11, and at page 71, lines 5-7 of the specification.

## **6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

- I. Whether Claims 12-14 satisfy the utility requirement of 35 U.S.C. §101.
- II. Whether Claims 12-14 satisfy the enablement requirement of 35 U.S.C. §112, first paragraph.

## **7. ARGUMENT**

### **Summary of the Arguments:**

#### **Issue I: Utility**

##### *The primary structure of Bolekine is consistent with its asserted utility*

Based on its primary sequence structure, Bolekin has been identified as a novel chemokine (Example 1, lines 2-22).

The specification teaches: “*Chemokines may be able to activate immune cells . . .*” and thus “*Bolekine may be useful in treating infections, as local administration of the polypeptide would stimulate immune cells already present at the site of infection and induce more immune cells to migrate to the site, thus removing the infection at a faster rate.*” (Page 71, lines 3-7)

##### *Experimental data provided in the specification confirm the asserted utility of Bolekine*

Indeed, experimental data provided in the specification are direct evidence of the immune stimulatory activity of Bolekine.

The specification discloses that Bolekine showed positive activity in the Mixed Lymphocyte Reaction (MLR) assay (see Example 10). The MLR is a well-established assay for

evaluating test compounds for their ability to stimulate T-lymphocyte proliferation *in vitro*. The assay protocol states that increases over control are considered positive with increases of greater than or equal to 180% being preferred. Example 10 shows that Bolekine, at a concentration of 124.00 nM, resulted in a 192.7% increase over control. Thus, the results set forth in Example 10 clearly establish that Bolekine is a stimulator of T cell proliferation. The specification explains that compounds which stimulate proliferation of lymphocytes in this assay, such as Bolekine, "*are useful therapeutically where enhancement of an immune response is beneficial.*" (Example 10, page 87, lines 5-6)

Example 11 discloses data obtained in the Vascular Permeability Assay. The data shows that Bolekine stimulates immune response and can induce mononuclear cell, eosinophil and PMN infiltration.

*Two expert Declarations under 37 C.F.R. 1.132 confirm the validity of the experimental data provided in the specification, and the utility of Bolekine in the treatment of medical conditions benefiting from immune stimulation*

Appellants have also submitted, with their Response dated October 26, 2005, two Declarations of Dr. Sherman Fong.

In one Declaration, dated June 16, 2004 (First Fong Declaration) Dr. Fong provides examples of important clinical applications for immune stimulants which have been shown to stimulate T-cell proliferation in the MLR assay, such as the chemokine IL-12, used in the treatment of melanoma. Dr. Fong concludes that "*a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant.*" The reference to a "PRO polypeptide" makes the statement generic, but the statement clearly applies to any polypeptide, including Bolekine, that shows to stimulate T-cell proliferation in the MLR assay.

In a second Declaration, dated August 27, 2004, Dr. Fong provides a detailed explanation of the Vascular Permeability Assay, and states that examples of utilities for molecules found positive in this assay include "*enhancing immune cell recruitment to sites of injury or infection.*"



The two Fong Declarations confirm the validity of the results obtained in the MLR and Vascular Permeability assays, and affirm the ability of Bolekine to enhance immune cell infiltration and to alleviate infection in a mammal, as claimed in claims 12-14 of the present application.

*At the effective filing date, one of ordinary skill would have clearly understood which conditions benefit from the enhancement of the immune response*

At the effective filing date of this application it was known that HIV-1 positive and AIDS patients benefit from the enhancement of their immune response which facilitates their defense against opportunistic infections (McElrath et al., Proc. Natl. Acad. Sci. USA 87(15):5783-7 (1990)). It was also known that other patients with opportunistic infections, such as multidrug-resistant tuberculosis benefit from immune stimulatory therapy (McDyer et al., J. Immunol. 158(1):492-500 (1997)), and the stimulation of immune response is beneficial in the treatment of various malignancies, including cutaneous T cell lymphoma (Zaki et al., J. Invest. Dermatol. 118(2):366-71 (2002)) and viral infections, such as genital and perianal warts (Tyring, S., Skin Therapy Lett. 6(6):1-4 (2001)). Accordingly, the statement at page 87, lines 5-6 of the specification that “[c]ompounds which stimulate proliferation of lymphocytes [in the MLR assay] are useful therapeutically where enhancement of an immune response is beneficial” would have been understood by those skilled in the pertinent art, without the need for any further explanation.

*Post-published evidence supports the asserted utility of Bolekine*

The asserted utility of Bolekine is also affirmed by post-published evidence. For example, Shurin et al., J. Immunol. 174(9):5490-5498 (2005)) confirm the MLR results disclosed in the present application by performing their own MLR assay and also finding that Bolekine (also known as CXCL14 or BRAK) is an immune cell chemoattractant.

Sleeman et al., Inter. Immuno. 12(5):677-689 (2000), found that CXL14/BRAK/Bolekine was chemotactic for a B cell lymphoblastoid line and a monocytic cell line, and that the murine form of CXL14/BRAK/Bolekine caused the migration of inflammatory cells *in vivo*.

### Conclusion

Thus, a large body of evidence, including the identification of Bolekine as a novel cytokine, the experimental data provided in the specification, and post-published scientific papers by independent research groups, all support the asserted utility of Bolekine and the methods claimed in the present application. Accordingly, the rejection of claims 12-14 on the ground of alleged lack of a credible, specific and substantial asserted utility is believed to be clearly erroneous, and should be overturned.

### Issue II: Enablement

Claims 12-14 stand rejected under 35 U.S.C. §112, first paragraph, allegedly "since the claimed invention is not supported by a specific, substantial and credible asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention." (Page 10 of the Office Action mailed 01/16/2007).

Appellants submit that, as discussed above, the MLR and the Vascular Permeability Assays, and the totality of evidence of record, clearly establish patentable utility for the claimed methods. Based on such a utility, one of skill in the art would know exactly how to use the claimed methods, without undue experimentation.

These arguments are all discussed in further detail below under the appropriate headings.

### ISSUE I: Claims 12-14 satisfy the utility requirement of 35 USC §101

Claims 12-14 stand rejected under 35 U.S.C. §101 because allegedly "the claimed invention is not supported by a credible, specific and substantial asserted utility or a well established utility for the isolated polypeptide" (Page 3 of the Office Action mailed 01/16/2007).

Appellants submit, for the reasons set forth below, that the specification discloses a credible, specific and substantial asserted utility for the Bolekine polypeptide of SEQ ID NO: 2, the claimed methods, which are based on such utility, meet the utility requirement of 35 U.S.C. §101.

**A. The Legal Standard for Utility**

According to 35 U.S.C. § 101:

Whoever invents or discovers any new and *useful* process, machine, manufacture, or composition of matter, or any new and *useful* improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title. (Emphasis added.)

In interpreting the utility requirement, in *Brenner v. Manson*,<sup>1</sup> the Supreme Court held that the *quid pro quo* contemplated by the U.S. Constitution between the public interest and the interest of the inventors required that a patent applicant disclose a "substantial utility" for his or her invention, i.e. a utility "where specific benefit exists in currently available form."<sup>2</sup> The Court concluded that "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion. A patent system must be related to the world of commerce rather than the realm of philosophy."<sup>3</sup>

Later, in *Nelson v. Bowler*,<sup>4</sup> the C.C.P.A. acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, even though they may not establish a specific therapeutic use. The court held that "since it is crucial to provide researchers with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility."<sup>5</sup>

In *Cross v. Iizuka*,<sup>6</sup> the C.A.F.C. reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results,

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<sup>1</sup> *Brenner v. Manson*, 383 U.S. 519, 148 U.S.P.Q. (BNA) 689 (1966).

<sup>2</sup> *Id.* at 534, 148 U.S.P.Q. (BNA) at 695.

<sup>3</sup> *Id.* at 536, 148 U.S.P.Q. (BNA) at 696.

<sup>4</sup> *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (C.C.P.A. 1980).

<sup>5</sup> *Id.* at 856, 206 U.S.P.Q. (BNA) at 883.

<sup>6</sup> *Cross v. Iizuka*, 753 F.2d 1047, 224 U.S.P.Q. (BNA) 739 (Fed. Cir. 1985).

i.e. there is a reasonable correlation there between."<sup>7</sup> The court perceived "No insurmountable difficulty" in finding that, under appropriate circumstances, "in vitro testing, may establish a practical utility."<sup>8</sup>

Furthermore, M.P.E.P. §2107.03 (III) states that:

"If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process."

Thus, the legal standard recognizes that *in vitro* or animal model data is acceptable to establish utility as long as the data is "reasonably correlated" to the pharmacological utility described.

The case law has also clearly established that applicants' statements of utility are usually sufficient, unless such statement of utility is unbelievable on its face.<sup>9</sup> The PTO has the initial burden to prove that applicants' claims of usefulness are not believable on their face.<sup>10</sup> In general, an Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope."<sup>11, 12</sup>

Compliance with 35 U.S.C. §101 is a question of fact.<sup>13</sup> The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration.<sup>14</sup> Thus, to overcome the presumption of truth that

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<sup>7</sup> *Id.* at 1050, 224 U.S.P.Q. (BNA) at 747.

<sup>8</sup> *Id.*

<sup>9</sup> *In re Gazave*, 379 F.2d 973, 154 U.S.P.Q. (BNA) 92 (C.C.P.A. 1967).

<sup>10</sup> *Ibid.*

<sup>11</sup> *In re Langer*, 503 F.2d 1380, 1391, 183 U.S.P.Q. (BNA) 288, 297 (C.C.P.A. 1974).

<sup>12</sup> *See also In re Jolles*, 628 F.2d 1322, 206 U.S.P.Q. 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 U.S.P.Q. 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 U.S.P.Q. 209, 212-13 (C.C.P.A. 1977).

<sup>13</sup> *Raytheon v. Roper*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984).

<sup>14</sup> *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d (BNA) 1443, 1444 (Fed. Cir. 1992).

an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

The well established case law is clearly reflected in the Utility Examination Guidelines (“Utility Guidelines”)<sup>15</sup>, which acknowledge that an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted “specific, substantial, and credible utility” or a “well-established utility.” Under the Utility Guidelines, a utility is “specific” when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that are to be diagnosed.

In explaining the “substantial utility” standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase “immediate benefit to the public” or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be “currently available” to the public in order to satisfy the utility requirement. “Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a ‘substantial’ utility.”<sup>16</sup> Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement,<sup>17</sup> gives the following instruction to patent examiners: “If the applicant has asserted that the claimed invention is useful for any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.”

**B. Proper Application of the Legal Standard**

Appellants rely on the identification of Bolekin as a novel cytokine, and data from the Mixed Leukocyte Reaction (MLR) assay and the Vascular Permeability Assay for patentable

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<sup>15</sup> 66 Fed. Reg. 1092 (2001).

<sup>16</sup> M.P.E.P. §2107.01.

<sup>17</sup> M.P.E.P. §2107 II (B)(1).

utility of the Bolekine polypeptide of SEQ ID NO: 2 and the claimed methods comprising the administration of Bolekine.

1. *Bolekine is a novel cytokine and its structure is consistent with the asserted utility*

Applicants have disclosed in the specification that Bolekine has the primary sequence structure of a chemokine, and state:

*“Chemokines may be able to activate immune cells, as shown with the CXC chemokines activation of neutrophils (Baggiolini et al. Adv. Immunology 1994; 55:97-179), and the non-CXC chemokines are mainly chemotactic for T lymphocytes. Bolekine may be useful in treating infection, as local administration of the polypeptides would stimulate immune cells already present at the site of infection and induce more immune cells to migrate to the site, thus removing the infection at a faster rate.”* (Specification, page 71, lines 3-7)

The positive results disclosed in Example 10 (MLR) and Example 11 (Vascular Permeability) demonstrate that Bolekine is active as a stimulator of the proliferation of stimulated T-lymphocytes, and enhances the infiltration of immune cells.

2. *Experimental data provided in the specification confirm the asserted utility of Bolekine*

*The MLR Assay*

The MLR is a well-established assay for evaluating test compounds, such as the Bolekine polypeptide, for their ability to stimulate T-lymphocyte proliferation *in vitro*, and consequently, for assessing the immune response of an individual. The MLR assay is well-described in standard textbooks, including, for example, *Current Protocols in Immunology*, unit 3.12; edited by J E Coligan, A M Kruisbeek, D H Marglies, E M Shevach, W Strober, National Institutes of Health, Published by John Wiley & Sons, Inc., which is referenced in Example 10, and the entire content of which is expressly incorporated by reference into the disclosure of the present application (see page 79, lines 3-4). In brief, in this method, an immune response results upon mixing T-cells from antigenically distinct individuals under cell culture conditions. An MLR reaction can be monitored quantitatively by, for example, following the incorporation of tritiated thymidine during DNA synthesis, or by observing blast formation, or by other methods well known in the art.

According to the specification, positive increases over control in this assay are considered to be positive results, with increases of greater than or equal to 180% being preferred. However, any value greater than control indicates a stimulatory effect for the test protein. Bolekine (SEQ ID NO:2) resulted in an increase of 192.7% over control when administered at a dose of 124.00 nm, and thus tested positive in this assay, using the described criteria. Example 10 further explains that compounds which stimulate proliferation of lymphocytes in this assay "*are useful therapeutically where enhancement of an immune response is beneficial.*" (Page 87, lines 5-6) Accordingly, Bolekine has utility in the treatment of conditions where the stimulation of lymphocyte proliferation is desirable.

*An expert Declaration under 37 C.F.R. 1.132 supports the validity of the results obtained in the MLR assay*

In further support of utility based upon the MLR assay, Appellants have submitted (with their Response dated 10/26/2005) the Declaration of Sherman Fong, Ph.D dated June 16, 2004 (First Fong Declaration). Dr. Fong is an inventor of the above-identified patent application, and an experienced scientist familiar with the MLR assay, which was used by him and others under his supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project, including Bolekine.

The First Fong Declaration explains how the MLR reaction was performed in the instant application using peripheral blood mononuclear cells (PBMCs), which contain responder T-cells, and allogenic, pre-treated (irradiated) PBMCs, which predominantly contained dendritic cells. Dr. Fong proceeds to explain (paragraph 7 of the Declaration) that dendritic cells are potent antigen-presenting cells that are able to "prime native T cells *in vivo*." Once activated by dendritic cells, the T-cells are capable of interacting with other antigen-presenting cells (B cells and macrophages) to produce additional immune responses from these cells.

As Dr. Fong states, the MLR assay of the present application,  
*is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to*

*respond to a particular antigen that may not have been immunologically active previously. (Paragraph 8 of the First Fong Declaration.)*

As Dr. Fong emphasizes, immunostimulants are important and highly desirable in the treatment of cancer and in enhancing the effectiveness of previously identified treatments for cancer. Supportive evidence also comes from teachings in the art such as Steinman *et al.* (submitted as Exhibit B with the Response filed September 9, 2004) who state that "...medicine needs therapies that enhance immunity or resistance to infections and tumors" (page 1, column 1, line 7; emphasis added).

In paragraph 9 of the First Fong Declaration, Dr. Fong provides examples of important clinical applications for immune stimulants which have been shown to stimulate T-cell proliferation in the MLR assay. As Dr. Fong explains,

*IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured in vitro and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].*

Dr. Fong concludes that (paragraph 10):

*It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant.*

Accordingly, the positive results obtained in this assay clearly establish the



immunostimulant utility for the polypeptides claimed in the present application, and the specification, in turn, enables one skilled in the art to use the compounds for the asserted purpose.

Appellants submit that the teachings of the specification are properly evaluated through the eyes of one skilled in the pertinent art at the effective filing date of the present application. In 1998 it was well known in the art, as it is today, that T-cells are highly important in the body's natural defense mechanisms for fighting infections. For example, viral infections, such as HIV infection, are well known to result in reduced T cell count. Indeed, the count of T-cell lymphocytes is a generally accepted measure of the extent and seriousness of HIV infection and resultant AIDS. Accordingly, stimulators of T-cell proliferation find utility in fighting viral infections, including retroviral infections, such as HIV infection or Epstein-Barr infection.

As discussed above, the legal standard is that *in vitro* results are acceptable to demonstrate utility. Further, the data derived from the MLR assay is "reasonably correlated" to the asserted pharmacological utility, because one of ordinary skill in the art would understand, based upon the discussion above, that molecules showing immunostimulant activity as measured in the MLR assay would be useful in the treatment of conditions where the stimulation of lymphocyte proliferation would be desirable, including viral infections such as HIV and Epstein-Barr. Accordingly, a valid case for utility has been made and would be considered credible by a person of ordinary skill in the art based on the MLR assay and the First Fong Declaration, including its Exhibits.

#### The Vascular Permeability Assay

This assay is also known as the Miles assay, and is described in Example 11. In brief, a protein that is believed to have a chemoattractant activity for immune cells is injected into the backs of hairless guinea pigs. Evans Blue dye is used as a tracer. If the molecule is an immune cell chemoattractant, then the immune cells will extravasate from the vasculature into the surrounding area. If the molecule is scored as positive, then the guinea pigs are sacrificed and the skin is sectioned for histopathologic evaluation. The skin sections are then evaluated for what types and how many immune cells have migrated to the site of injection. The Bolekine polypeptide was strongly positive in this assay.

An expert Declaration under 35 U.S.C. 1.132 supports the validity of the data obtained in the Vascular Permeability Assay

To further support the validity of this assay to identify proinflammatory molecules and molecules enhancing immune cell infiltration, Applicants submitted a Declaration of Sherman Fog, dated 8/27/2004 (Second Fong Declaration).

In the Second Fong Declaration, Dr. Fong further discusses the skin vascular permeability assay, as was used in the instant application, the mechanism for vascular permeability and how this assay identifies proinflammatory molecules, how the assay and its modifications have been widely used in the art, by several investigators, to identify various well-established proinflammatory molecules such as Vascular Endothelial Growth Factor (VEGF) and others. Dr. Fong, who oversaw and participated in this experimental work, states in his declaration that the results were further analyzed by histopathological examination to rule out inflammation due to endothelial cell damage or mast cell degranulation. Dr. Fong clearly states that the vascular permeability increase that was observed due to the tested PRO polypeptides (including Bolekine) was not due to histamine release or endothelial cell damage. Thus, based on the positive score obtained by Bolekine in this assay, Bolekine can be clearly identified as being capable of enhancing recruitment to the sites of injury or infection (Second Fong Declaration, paragraph 14).

3. At the effective filing date of the application, one of ordinary skill would have clearly understood which conditions benefit from the enhancement of immune response

As explained in Appellants' response dated October 17, 2006, at the effective filing date of this application it was known that HIV-1 positive and AIDS patients benefit from the enhancement of their immune response which facilitates their defense against opportunistic infections (McElrath et al., Proc. Natl. Acad. Sci. USA 87(15):5783-7 (1990)). It was also known that other patients with opportunistic infections, such as multidrug-resistant tuberculosis benefit from immune stimulatory therapy (McDyer et al., J. Immunol. 158(1):492-500 (1997)), and the stimulation of immune response is beneficial in the treatment of various malignancies, including cutaneous T cell lymphoma (Zaki et al., J. Invest. Dermatol. 118(2):366-71 (2002)) and

viral infections, such as genital and perianal warts (Tyring, S., Skin Therapy Lett. 6(6):1-4 (2001)). Accordingly, the statement at page 87, lines 5-6 of the specification that “[c]ompounds which stimulate proliferation of lymphocytes [in the MLR assay] are useful therapeutically where enhancement of an immune response is beneficial” would have been understood by those skilled in the pertinent art, without the need for any further explanation.

4. Evidence published after the priority date of the present application supports the asserted utility

Bolekine is also known as CXCL14 or BRAK. Several papers submitted with Appellant’s response dated October 26, 2005 confirm the results and asserted utilities disclosed in the present application.

Thus, for example, in a paper by Shurin et al. (*J. Immunol.* 174(9):5490-5498 (2005)) the authors confirm the MLR results disclosed in the present application by performing their own MLR assay and also finding that CXCL14/BRAK/Bolekine is an immune cell chemoattractant (see page 5495).

Sleeman et al., *Inter. Immuno.* 12(5):677-689 (2000), found that CXL14/BRAK/Bolekine was chemotactic for a B cell lymphoblastoid line and a monocytic cell line (see Figure 8, page 684, and Results, page 686). The murine form of CXL14/BRAK/Bolekine also caused the migration of inflammatory cells *in vivo*. Sleeman et al. injected the murine form of CXL14/BRAK/Bolekine into the foot pad of mice, and found that this caused infiltration of mononuclear and polymorphonuclear cells.

These results further confirm the asserted utility of the Bolekine polypeptide, and of the methods claimed in the present application.

C. A prima facie case of lack of utility has not been established

As a preliminary matter, Appellants submit that, as discussed above, the evidentiary standard to be used throughout *ex parte* examination of a patent application is a preponderance of the totality of the evidence under consideration. Accordingly, Appellants submit that in order to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would

doubt the truth of the assertion of utility. With respect to asserted therapeutic utilities based upon *in vitro* data, an applicant "does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty."<sup>18</sup> The law requires only that one skilled in the art should accept that such a correlation is more likely than not to exist. Appellants respectfully submit that when the proper evidentiary standard is applied, a correlation must be acknowledged. It is also well established that utility, just as other indicia of patentability, must be evaluated on the totality of evidence of record.

The Examiner's assertions in support of the lack of utility finding can be briefly summarized as follows:

(1) There is "no information regarding the correlation of the results of the mixed lymphocyte reactions (results from Example 10) of the Bolekine polypeptide to any real life diseases," and the "specification fails to teach where an enhancement of an immune response is beneficial and therapeutically useful." (Office Action of 01/16/2007, page 3).

(2) The "specification does not disclose how the induction of inflammation (results from Example 11) is beneficial and therapeutically useful." (Office Action of 01/16/2007, page 3).

(3) "The assertion that the claimed invention could be useful for the treatment of conditions where the enhancement of the immune response would be beneficial is not specific since there are many of such conditions, and it is not predictable of which conditions the claimed invention may function, if any." (Office Action of 01/16/2007, page 4). "The Examiner understands that there are conditions where the enhancement of an immune response is beneficial," but the "instant specification fails to teach those conditions specific to Bolekine polypeptides." (Office Action of 01/16/2007, page 6, emphasis omitted)

(4) Example 10 is deficient since it does not provide "any data or evidence of the results of the assay," including "statistical significance." (Office Action of 01/16/2007, page 4).

(5) The First Fong Declaration, concerning the MLR assay, is not persuasive, since authors of the publications cited in the Declaration (Gubler, Dziarski) employed other assays,

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<sup>18</sup> M.P.E.P. §2107.03.

outside the MLR assay, to characterize the proteins tested, while the specification of the present application does not provide a “well characterized structure/function analysis” for the Bolekine protein. (Office Action of 01/16/2007, page 9)

(6) The Second Fong Declaration, concerning the Vascular Permeability Assay (also known as the Miles assay), is not persuasive since the assay is “useful as a preliminary screen for potential proinflammatory molecules,” but “further work must be done subsequent to a positive result in a Miles assay to determine if and how a molecule may be useful as a proinflammatory.” (Office Action of 01/27/2006, page 5) The Examiner additionally notes that (i) “[b]asic irritants, such as lye, would test positive in the Miles assay,” (ii) the Second Fong Declaration is not specific for Bolekine, (iii) with regard to Dr. Fong’s statement that the PRO polypeptides that tested positive in this assay were further analyzed by histopathological examination to rule out inflammation due to endothelial cell damage or mast cell degranulation, the Examiner notes that “the Declarant is not entirely correct with respect to the facts.” (Office Action of 01/27/2006, pages 8-9). In the Office Action of 01/16/2007, the Examiner explains that the latter statement “was made because those teachings were not disclosed in the specification as originally filed.” (Page 9)

The Examiner concludes that “the scientific reasoning and evidence as a whole indicates that the rejection should be maintained.” (Office Action of 01/16/2007, page 9) Contrary to the Examiner’s assertion, the evidence of record as a whole overwhelmingly supports the asserted utility, and indicates that the present rejection should be overturned.

As discussed above, the asserted utility (the immune stimulatory activity of Bolekine) is consistent with its primary structure as a cytokine, has been supported experimentally in well established and widely used assays, has been confirmed in independent experiments by independent research groups after the effective filing date of the present application, and is known to result in important, real-life applications, such as the treatment of various infections, as disclosed in the specification, and as it was known in the art at the effective filing date of the present application.

The Examiner's finding that the two Fong Declarations are not persuasive is particularly troubling, and lacks any objective rational basis. The fact that in the course of developing therapeutic agents with immune stimulatory activity that were first discovered in the MLR assay, further confirmatory tests were run is irrelevant for patentability determination. In the course of developing a drug, it is of course customary and necessary to confirm an original activity of a drug candidate in a series of *in vitro* and *in vivo* assays. The FDA will only approve a drug, if the candidate continues to show the asserted activity in such assays, including human clinical trials, and does not show unacceptable levels of toxicity or side-effects. However, the Patent Office is not the FDA; and it is well established that patentable utility can be based on *in vitro* assays that have acquired general acceptance in the pertinent art.

Thus, In *Cross v. Iizuka*, *supra* the C.A.F.C. recognized that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, i.e. there is a reasonable correlation there between."<sup>19</sup> The court perceived "No insurmountable difficulty" in finding that, under appropriate circumstances, "in vitro testing, may establish a practical utility."<sup>20</sup>

Furthermore, M.P.E.P. §2107.03 (III) states that:

"If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process."

It is submitted that the wide-spread use of the MLR assay and the post-published data confirming the immune stimulatory activity of Bolekine jointly clearly establish that the MLR assay is predictive of an *in vivo* immune stimulatory activity. Furthermore, the disclosure of the specification and the art in general clearly enable a person skilled in the art to identify medical conditions that benefit from the enhancement of immune cell infiltration (claims 12-13) and

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<sup>19</sup> *Id.* at 1050, 224 U.S.P.Q. (BNA) at 747.

<sup>20</sup> *Id.*

would accept that Bolekine is expected to be efficacious alleviating infection (claim 14).

The case law has clearly established that in considering affidavit evidence, the Examiner must consider all of the evidence of record anew.<sup>21</sup> "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument."<sup>22</sup> Furthermore, the Federal Court of Appeals held in *In re Alton*, "We are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner."<sup>23</sup> Applicants also respectfully draw the Examiner's attention to the Utility Examination Guidelines<sup>24</sup> which state, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered." The statement in question from an expert in the field (the Fong Declaration) states that "(i)t is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity of at least 180% of the control is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant." Therefore, barring evidence to the contrary regarding the above statement in the Fong Declaration, this rejection is improper under both the case law and the Utility guidelines.

Similarly, the Examiner's statement that the second Fong Declaration is "not entirely correct with respect to the facts" is misplaced. Dr. Fong has first hand knowledge about how the test was performed. While not all details might have been disclosed in the specification, the Examiner has no basis for disregarding or questioning the accuracy of Dr. Fong's statements. On the contrary, any disagreement between the description provided in the specification and Dr.

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<sup>21</sup> *In re Rinehart*, 531 F.2d 1084, 189 U.S.P.Q. 143 (C.C.P.A. 1976); *In re Piasecki*, 745 F.2d 1015, 226 U.S.P.Q. 881 (Fed. Cir. 1985).

<sup>22</sup> *In re Alton*, 37 U.S.P.Q.2d 1578, 1584 (Fed. Cir. 1996) (quoting *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992)).

<sup>23</sup> *In re Alton*, *supra*.

<sup>24</sup> Part IIB, 66 Fed. Reg. 1098 (2001).

Fong's statements should be resolved by accepting Dr. Fong's description. Thus, both the MLR and the Vascular Permeability Assays should be read in the light of the two Fong Declarations, which provide further details of the assays and confirm the meaning and validity of the results obtained in the assays.

Finally, the Examiner's assertion that the statement that Bolekine is beneficial where the enhancement of immune response is beneficial is not specific "since there are many such conditions, and it is not predictable of which conditions the claimed invention may function" (Office Action of 1/16/2007) is believed to be misplaced. First of all, utility must be assessed with regard to the utility claimed. Pending claim 14 is directed to the alleviation of infection in a mammal by administering a Bolekin polypeptide. Thus, claim 14 recites a specific condition, also taught in the specification and by general knowledge in the art at the priority date of this application, and thus the Examiner's rationale does not apply.

Similarly, claims 12-13, that are drawn to the enhancement of the infiltration of immune cells, are supported by at least the same condition (alleviation of infection), which should be sufficient for establishing a patentable utility.

In summary, Appellants respectfully submit that the Examiner has not shown that it is it is more likely than not that one of ordinary skill in the art would doubt the truth of Appellants' assertion of utility. And the rejection of claims 12-14 for alleged lack of utility should be overturned.

**ISSUE II: Claims 12-14 satisfy the enablement requirement of 35 USC §112, first paragraph.**

Claims 12-14 stand rejected under 35 U.S.C. §112, first paragraph, allegedly "since the claimed invention is not supported by either a credible, specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention." (Page 10 of the Office Action mailed 01/16/2007).

In this regard, Appellants refer to the arguments and information presented above in response to the issue of utility, wherein those arguments are incorporated by reference herein. Appellants submit that, as discussed above, the totality of evidence, including the identification of Bolekine as a cytokine, the MLR and Vascular Permeability Assays, the two Fong Declarations,



general knowledge in the art at the effective filing date of the present application, and post-published evidence, clearly establish the utility of Bolekine in the enhancement of the infiltration of immune cells and, specifically alleviation of infection in a mammal. Based on such a utility, one of skill in the art would know exactly how to use Bolekine in the methods claimed in rejected claims 12-14, and the Examiner's rejection under this section should be overturned.

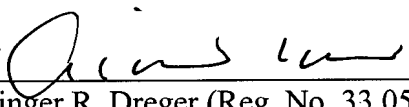
### **CONCLUSION**

For the reasons given above, Appellants submit that claims 12-14 meet the requirements of 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph. Accordingly, reversal of all the rejections of claims 12-14 is respectfully requested.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 08-1641 (referencing Attorney's Docket No. 39780-1192-2C1).

Respectfully submitted,

Date: November 13, 2007

By:   
Ginger R. Dreger (Reg. No. 33,055)

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## 8. CLAIMS APPENDIX

### Claims on Appeal

12. (Previously presented) A method of enhancing the infiltration of immune cells in a mammal, comprising administering to said mammal an effective amount of Bolekine polypeptide as shown in Figure 2 (SEQ ID NO: 2).

13. (Previously presented) The method of Claim 12, wherein said immune cells are mononuclear cells, eosinophils, or polymorphonuclear neutrophils (PMNs).

14. (Previously presented) A method of alleviating infection in a mammal comprising administering an effective amount of Bolekine polypeptide as shown in Figure 2 (SEQ ID NO: 2).

## 9. EVIDENCE APPENDIX

1. Declaration of Sherman Fong, Ph.D. under 35 C.F.R 1.132, dated June 16, 2004, with attached Exhibits A-E ("First Fong Declaration"):

A. Current Protocols in Immunology, Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12

B. Steinman, R.M., "The dendritic cell advantage: New focus for immune-based therapies," *Drug News Perspect.* **13**:581-586 (2000).

C. Gubler, U. et al., "Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor," *Proc. Natl. Acad. Sci. USA* **88**:4143-4147 (1991).

D. Peterson, A.C. et al., "Immunization with melan-A peptide-pulsed peripheral blood mononuclear cells plus recombinant human interleukin-12 induces clinical activity and T-cell responses in advanced melanoma," *J. Clin. Oncol.* **21**:2342-2348 (2003).

E. Thurner, B. et al., "Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma," *J. Exp. Med.* **190**:1669-1678 (1999).

The Declaration of Dr. Sherman Fong and attached Exhibits A-E were submitted with Appellants' response filed September 9, 2004, and noted as entered into the record in the Office Action mailed October 20, 2004.

2. Declaration of Sherman Fong, Ph.D. under 35 C.F.R 1.132, dated August 27, 2004, with attached Exhibits A-I ("Second Fong Declaration"):

Miles A. A. and Miles E.M., *J. Physiol.*, 118:228-254 (1952).

Regulation of Leukocyte Movement

Baggiolini et al., *Adv. Immunology* 55:97-179 (1994).

Strieter et al., *J. Biol. Chem.* 240:27348-27357 (1995).

Udaka et al., *Proc. Soc. Exp. Biol. Med.* (133) 1384-1387 (1970).

- Hirahara K., et al., *Thrombosis Res.* 71(2):139-148 (1993).
- Senger D.R., et al., *Science* (219) 983-985 (1983).
- Yeo K.T., *Clin. Chem.* (38) 71-75 (1992).
3. Shurin et al., *J. Immunol.* 174(9):5490-5498 (2005).
  4. Sleeman et al. *Inter. Immuno.* 12(5):677-689 (2000).
  5. McElrath et al., *Proc. Natl. Acad. Sci. USA* 87:5783-5787 (1990).
  6. McDyer et al., *J. Immunol.* 158:492-500 (1997).
  7. Zaki et al., *J. Invest. Dermatol.* 118:366-371 (2002).
  8. Tying et al., *Skin Therapy Letter* 6:1-4 (2001).
  9. Dziarski, *J. Immunol.* 143:456-465 (1989).

**10. RELATED PROCEEDINGS APPENDIX**

None.

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11/12/07 11:54 AM (39780.1192)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:

Docket No.:

Serial No.:

Group Art Unit:

Filing Date:

Examiner:

For:

**DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.
2. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.
3. My scientific Curriculum Vitae is attached to and forms part of this Declaration.
4. I am familiar with the Mixed Lymphocyte Reaction (MLR) assay, which has been used by me and others under my supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.
5. The MLR assay is a well known and widely used proliferative assay of T-cell function, the basic protocols of which are described, for example, in Current Protocols in Immunology Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12. (Exhibit A). This publication is incorporated by reference in the description of the MLR protocol in the present application.

6. The T-lymphocytes or "T-cells" of our immune system can be induced to proliferate by a variety of agents. The MLR assay is designed to study a particularly important induction mechanism whereby responsive T-cells are cultured together (or "mixed"), with other lymphocytes that are "allogeneic", e.g. lymphocytes that are taken from different individuals of the same species. In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells. It is known that the dendritic cell population among the PBMCs are differentially affected by irradiation. At low doses (500-1000 Rad), the proliferation of most cells, including the B cells in the PBMCs, is preserved, however, at doses above 2000 Rad, this function of B cells is abolished. Dendritic cells on the other hand, maintain their antigen presentation function even at a 3000 Rad dose of radiation. (See, e.g. Current Protocols in Immunology, *supra*, at 3.12.9). Accordingly, under the conditions of the MLR assay used to test the PRO polypeptides of the present invention, the stimulator PBMCs remaining after irradiation are essentially dendritic cells.
7. Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naive T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells. For further details about the properties and role of dendritic cells in immune-based therapies see, e.g. Steinman, Drug News Perspect. 13(10):581-586 (Exhibit B).
8. The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

9. Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Turner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].

10. It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Some PRO polypeptides do the reverse, and give inhibition of T-cell proliferation in the MLR assay. It is my considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.

Dated: 6/16/04

By: Sherman Fong  
Sherman Fong, Ph.D.



## **Sherman Fong, Ph.D.**

Senior Scientist

Department of Immunology

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### Education:

1978 - 1980 Postdoctoral Fellow in Immunology, Research Institute of Scripps Clinic,  
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology, University of California at  
San Francisco, San Francisco, California

1970 - 1975 Ph.D. in Microbiology, University of California at  
Davis, California

1966 - 1970 B.A. in Biology/Microbiology, San Francisco State  
University, San Francisco, California

### Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc.  
South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological  
Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco,  
California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical  
Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research,  
Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical  
Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical  
Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology  
and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Research Grant, NIH (1985)

Recipient: P.I., Research Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Committee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmacological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the **Biochemical Subteam for Protropin® Project team.**

Served as Immunologist on the **Met-less hGH and Dnase project teams**, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in 1989-1990 on the **CD4-IgG project team** carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte  $\beta 2$  integrins. Provided preclinical scientific data to **Anti-CD18 project team**

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule  $\alpha 4\beta 1$  integrin-antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped establish and responsible for maintaining current research collaboration with Roche-Nutley. Project transferred to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against  $\alpha 4\beta 7$  integrin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn's disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for  $\alpha 4\beta 7$ /MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc reviewer** on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as Scientific advisor with staff of the **Business Development** Office on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The  $\beta 2$  Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process -- Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process -- Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin  $\alpha 4 \beta 7$  Attenuates Inflammatory Bowel Diseases", in Symposium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.

U.S. Patent Number 5,068,177: Anti-idiotypic Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.

U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.

U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colitis, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen

U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood

U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood. U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura

US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood  
US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

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# Proliferative Assays for T Cell Function

UNIT 3.12

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (UNIT 3.10) or cytotoxicity (UNIT 3.11)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (UNIT 3.13) or T cell hybridomas (UNIT 3.14).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. The first basic protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR- $\gamma\delta$ , or anti-TCR- $\alpha\beta$  monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the V regions of  $\beta$  chains of the TCR (anti-V $\beta$ ) or with enterotoxins specific for certain V $\beta$ -chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). The first alternate protocol describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. The second alternate protocol describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). The first support protocol describes the preparation and use of T cell-depleted accessory or stimulator cells and the second support protocol describes methods for blocking accessory cell proliferation. Finally, the second basic protocol describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed *in vivo*.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in UNIT 3.10. Related assays for use with human peripheral blood lymphocytes are described in UNIT 7.9.

**NOTE:** All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

## ACTIVATION OF UNPRIMED T CELLS

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T cell proliferation is dependent on the presence of non-T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non-T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell-independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [ $^3$ H]thymidine between stimulated and control cells.

### BASIC PROTOCOL

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3.12.1



**Table 3.12.1** Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent <sup>a</sup>	Source/ cat. no. <sup>b</sup>	Concentration	Accessory cells <sup>c</sup>	Mode of action, etc.
PMA	SIG P8139	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL 407950	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL 100105	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD HA16	1-5 µg/ml	Yes	Indirect TCR cross-linking
Con A	PH 17-0450-01	1-10 µg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 µg/ml	Yes <sup>c</sup>	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 µg/ml	Yes <sup>c</sup>	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 µg/ml	Yes <sup>c</sup>	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2 <sup>c</sup>	PG MM-Vβ-TCR-1	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6 <sup>c</sup>	PG RM-Vβ-TCR-2	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT AT101	1-10 µg/ml	Yes <sup>c</sup>	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT BT202; SIG S4881	1-100 µg/ml	Yes <sup>c</sup>	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT ET404	1-10 µg/ml	Yes <sup>c</sup>	Vβ-11,15,17-receptor specificity

<sup>a</sup>Abbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus enterotoxins* A, B, & E.

<sup>b</sup>Supplier addresses and phone numbers are provided in APPENDIX 5. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

<sup>c</sup>When using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

## Materials

Complete RPMI-5 and RPMI-10 media (APPENDIX 2)

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (UNIT 3.1)

Activating agent(s) (Table 3.12.1)

Phosphate-buffered saline (PBS; APPENDIX 2)

Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

[<sup>3</sup>H]thymidine (APPENDIX 3)

15- and 4-ml disposable, polystyrene conical tubes with screw caps

Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)

1-, 5-, and 10-ml disposable polystyrene pipets

96-well flat- or round-bottom microtiter plates with lids (Costar #3596 or #3799)

25- to 100- $\mu$ l single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (UNIT 1.9), preparing single-cell suspensions (UNIT 3.1), and counting, labeling, and harvesting cells (APPENDIX 3)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in UNIT 3.1.

*The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4<sup>+</sup> or CD8<sup>+</sup>) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.*

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~1000 rpm (200  $\times$  g), room temperature, and discard supernatant.
3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to ~10<sup>6</sup> cells/ml with complete RPMI-10.

*While this concentration (1  $\times$  10<sup>6</sup> cells/ml or 2  $\times$  10<sup>5</sup> cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8  $\times$  10<sup>5</sup> cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non-T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers (0.1, 0.5, and 1.0  $\times$  10<sup>5</sup>) of syngeneic spleen (accessory) cells in 0.1 ml to 2  $\times$  10<sup>5</sup> T cells in 0.1 ml (see first support protocol). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.*

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from 1 mg/ml stock solutions—e.g., 100, 30, 10, and 3  $\mu$ g/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1  $\mu$ g/ml A23187 (or 4  $\mu$ g/ml ionomycin) in PBS.

*If MAb in supernatant or ascites form are being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.*

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See Table 3.12.1 for V $\beta$  specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enterotoxins and their specificities.

5. Add 20  $\mu$ l of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20  $\mu$ l of PBS only. Add 20  $\mu$ l PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

*A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.*

6. To the wells of the 96-well microtiter plate containing activating agent, add  $2 \times 10^5$  cells in 0.2 ml.

7. Place microtiter plates in a humidified 37°C, 5% CO<sub>2</sub> incubator for 2 to 4 days.

*Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see critical parameters).*

8. Add [<sup>3</sup>H]thymidine to each well. Return the plates to CO<sub>2</sub> incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in  $\beta$  scintillation counter.

- 9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as " $\Delta$  cpm."

- 9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to as "SI" (stimulation index).

*The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications,  $\Delta$  cpm rather than SI values are preferred.*

#### ALTERNATE PROTOCOL

#### ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor-bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in the basic protocol should be followed.

### Additional Materials

PBS (APPENDIX 2), room temperature and 4°C

1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) or 1 mg/ml purified anti-V $\beta$  or anti-TCR- $\gamma\delta$  MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1  $\mu$ g/ml—using room temperature PBS.

*Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.*

*The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.*

*Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FCS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.*

2. Add 30  $\mu$ l of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30  $\mu$ l PBS only.

*A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.*

*Most often, optimal responses are seen with 10  $\mu$ g/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is  $\sim$ 2 to 3  $\mu$ g (A.M.K., unpub. observ.).*

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

*During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.*

4. Prepare responder cell suspensions as in steps 1 to 3 of the basic protocol.

*Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell-independent. However, the presence of non-T accessory cells does not interfere with the proliferative response.*

5. Wash the wells of the incubated plates by adding 200  $\mu$ l cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add  $\sim$ 2  $\times$  10<sup>5</sup> cells in 0.2 ml.

*If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100  $\mu$ l PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to  $\leq$ 4 day periods. The PBS should be removed before the cells are added.*

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Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of the basic protocol, but incubate cultures for 2 to 3 days before adding [ $^3\text{H}$ ]thymidine.

*Kinetic assays should be performed to determine the optimum culture period.*

#### ALTERNATE PROTOCOL

### T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [ $^3\text{H}$ ]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see support protocols).

#### Additional Materials

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (UNITS 1.9 & 3.1) or purified T cells or T cell subpopulations (UNITS 3.1-3.6)

Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at *H-2* or *ML* loci, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

1. Prepare responder cell populations as in steps 1 to 3 of the basic protocol. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

*To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following:  $0.5$ ,  $1$ ,  $2$ , and  $4 \times 10^5$  cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.*

*For thymocytes, it may be necessary to use  $8 \times 10^5$  cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be  $1 \times 10^5$  and the doses in between would be  $2$  and  $4 \times 10^5$ . Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.*

2. To a 96-well microtiter plate, add  $5 \times 10^4$  to  $4 \times 10^5$  responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

*Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (UNIT 3.11), which is performed in 24-well microtiter plates. For example, cells can be diluted to  $4 \times 10^6$  cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.*

3. Prepare a single-cell suspension of irradiated or mitomycin C-treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

*The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from  $0.5$ - $4 \times 10^5$ , test stimulator cells at densities of  $2$ ,  $4$ , and  $8 \times 10^6$ /ml (i.e.,  $2$ ,  $4$ , and  $8 \times 10^5$ /well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If*

*highly purified T cells are used as the responder population, it is therefore not necessary to supplement the cultures with non-T accessory cells syngeneic to the responder T cells.*

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells. Values obtained from these controls reflect “background” proliferation values (see step 9 of basic protocol). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow steps 7 to 9 of the basic protocol, but incubate the cultures for 3 to 6 days.

*Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see critical parameters).*

### **DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS**

Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (second support protocol) can produce cytokines. In the following steps, T cell-depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non-T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell-depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in UNIT 3.7 does not deplete T cells and therefore is not recommended here; the method described in UNIT 3.15 leads to higher levels of enrichment that are not required in the protocols presented here.

#### **Additional Materials**

- Spleen cells from nonimmunized mice
- Hanks balanced salt solution (HBSS; APPENDIX 2)
- Low-Tox rabbit complement (Cedarlane #CL3051), reconstituted with ice-cold distilled water and filter-sterilized
- Anti-Thy-1.2 ascites (HO-13-4; ATCC #TIB 99) or anti-Thy-1.1 ascites (HO-22-1; ATCC #TIB 100; alternatively, see Table 3.4.1 for other anti-Thy-1 MAb and UNIT 2.6 for production of ascites)
- 70% Percoll solution (UNIT 3.8 and reagents and solutions)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

*The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.*

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25  $\mu$ l anti-Thy-1 ascites.

*If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.*

### **SUPPORT PROTOCOL**

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*The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.*

3. Incubate the mixture at 45 min in a 37°C water bath.
4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × g), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.
5. Count viable cells (APPENDIX 3) and resuspend in complete RPMI-10 or PBS for inactivation as in the second support protocol, or in HBSS to prepare low-density accessory cells (see below).

*The T cell-depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.*

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell-depleted spleen cells from step 5 in HBSS at  $20 \times 10^6$  cells/ml.
7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.
8. Spin 13 min in H-1000B rotor at 3000 rpm (1900 × g), room temperature.
9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.
10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to the second support protocol.

*The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.*

## SUPPORT PROTOCOL

### BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in the basic protocol, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see APPENDIX 1, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by  $\gamma$  irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

#### Mitomycin C Treatment

##### Additional Materials

Mitomycin C (Sigma #M-0503; store in dark)

1. In a 15-ml aluminum foil-wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

*Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.*

2. Prepare spleen cell suspension as described in steps 1 and 2 of the basic protocol at a concentration of  $5 \times 10^7$  cells/ml in PBS.
3. Add mitomycin C to a final concentration of 50  $\mu$ g/ml (100  $\mu$ l/ml of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.
4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm ( $300 \times g$ ). Discard supernatant and repeat washing procedure two more times.

*Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.*

5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to step 6 of the basic protocol.

### **Irradiation Treatment**

Prepare a spleen cell suspension as described in steps 1 to 3 of the basic protocol, at a final concentration of  $5\text{--}10 \times 10^6$  cells/ml in complete RPMI-10. Using a source of ionizing irradiation ( $^{60}\text{Co}$  or  $^{137}\text{Cs}$   $\gamma$ -irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses  $>2000$  rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of  $<1000$  rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

### **ACTIVATION OF PRIMED T CELLS**

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require in vivo immunization followed by in vitro stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary in vitro responses (i.e., MHC antigens) can be obtained by in vivo priming. Multiple immunizations usually elevate in vitro responses.

To immunize animals for in vitro secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freund's adjuvant (UNIT 2.5). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For

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strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with  $1-5 \times 10^7$  cells that express the antigen. Immunization protocols are described in UNIT 1.6.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (UNIT 3.14) and T cell hybridoma preparation (UNIT 3.13).

### Materials

Complete RPMI-10 medium (APPENDIX 2)

Responder cells: Purified T cells isolated from lymph nodes (UNITS 3.1-3.6) of in vivo primed mice

Antigen: 1 mg/ml sterile protein antigen(s) (UNIT 3.13), in PBS or suspension of irradiated or mitomycin C-treated stimulator cells expressing alloantigens at  $8 \times 10^6$  cells/ml (UNIT 3.11, support protocol) in complete RPMI-10 medium (APPENDIX 2)

Accessory cells: suspension of irradiated or mitomycin C-treated (or T cell-depleted) spleen cells syngeneic to the responding T cells at  $5 \times 10^6$  cells/ml in complete RPMI-10 medium

4-ml conical tubes

96-well flat-bottom microtiter plates with lids

1. Follow steps 1 to 3 of the first basic protocol for preparation of responder cells.
2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

*The following dilutions are recommended: 100, 10, 1, and 0.1  $\mu$ g/ml protein antigens and 8, 4, 2, and  $1 \times 10^6$  cells/ml of stimulator cells in complete medium.*

3. Add antigens to 96-well flat-bottom microtiter plates, at 30  $\mu$ l/well for protein antigens or 100  $\mu$ l/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

*By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.*

4. Add responder T cells in 0.1 ml to each well.

*Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non-T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add  $1-2 \times 10^5$  cells per well and proceed to step 6.*

5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at  $5 \times 10^5$  cells per well.

*Purified T cells require an exogenous source of accessory non-T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.*

6. Proceed as in steps 7 to 9 of the basic protocol.

*Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.*

## REAGENTS AND SOLUTIONS

### *Percoll solution*

#### *Diluent:*

45 ml 10× PBS, pH 7.4 (APPENDIX 2)

3 ml 0.6 M HCl

132 ml H<sub>2</sub>O

Filter sterilize

#### *70% Percoll solution:*

63 ml Percoll (Pharmacia LKB #170891-01)

37 ml sterile diluent (above)

Final osmolarity should be 310 to 320 osM

## COMMENTARY

### Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in UNIT 3.11 or the lymphokine production assays in UNITS 3.15 & 6.3. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variously reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "bystander" cells, and will be severely affected by the function of non-T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in UNITS 3.15 & 6.3. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (UNIT 6.1) or by flow cytometry (UNIT 5.4). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

### Critical Parameters and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO<sub>2</sub> level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in the first basic protocol to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR- $\alpha\beta$  or - $\gamma\delta$ , as well as anti-V $\beta$  antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (V) gene segment of the TCR. Different toxins have affinities for different V $\beta$  chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the

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mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (UNIT 3.15). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or “second signals” are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of UNITS 3.8, 3.11, & 3.13. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C-treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see first support protocol) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be

bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [ $^3$ H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and “cold” thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [ $^3$ H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [ $^3$ H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [ $^3$ H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal calf serum (FCS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. Our laboratory has found consistently better responses in round-bottom plates when

thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [<sup>3</sup>H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in APPENDIX 2), presumably because these media contain additional nutrients and have an osmolality more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO<sub>2</sub> will be sufficient, but for other media, a 7.5% CO<sub>2</sub> concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO<sub>2</sub> concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell prolifer-

ation. Addition of [<sup>3</sup>H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

### Anticipated Results

For proliferative assays described in the basic protocol, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-Vβ), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

### Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in critical parameters. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

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*Both of the above describe how specific proliferation of antigen-primed T cells can be measured.*

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*Details the MLC proliferation assay.*

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Exhibit 1

*Dendritic cells are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.*

# The Dendritic Cell Advantage: New Focus For Immune-Based Therapies

by Ralph M. Steinman

The focus of immune therapeutics has been on lymphocytes, the cellular mediators of immunity, and the suppression of lymphocyte function. The drug ciclosporin (cyclosporine) is an excellent and successful example. However, medicine needs therapies that enhance immunity or resistance to infections and tumors. Medicine also needs strategies, whether suppressive or enhancing, that are specific to the disease-causing stimulus or antigen. In contrast to lymphocytes, dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. DCs capture antigens and then initiate and control the activities of lymphocytes, including the development of resistance to infections and tumors (reviewed in references 1-3).

## Summary

Dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. The best-studied function of DCs is to convert antigens into immunogens for T cells. The "DC advantage" entails a myriad of functions. DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity. Another special feature of DCs is their location and movement in the body; DCs are stationed at surfaces where antigens gain access to the body. The events that make up the life history of DCs are now being unraveled in molecular terms. As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent. © 2000 Prous Science. All rights reserved.

The controlling role of DCs is best known for thymus-dependent lymphocytes or T cells which are important in many diseases, the most poignant being the AIDS epidemic (Table I). DCs were identified in a few laboratories that were focusing on the induction of immunity from resting T cells. It was noted that immune tissues (spleen, lymph nodes, lymph. blood) had a small fraction of cells with unusual

"tree-like" or "dendritic" processes. These distinctive cells had not been recognized previously and they proved to have distinct functions. Most importantly, DCs were potent inducers of immunity even in animals, not just the test tube, and now even in patients (reviewed in references 1-3).

The DC field was held back by the fact that there were so few cells relative

TABLE 1: HUMAN DISEASES THAT INVOLVE T CELLS

- Rejection of organ transplants and graft-vs.-host disease in bone marrow transplantation
- Resistance to many infections including vaccine design
- Vaccines against tumors and immune therapies for existing tumors
- Allergy
- AIDS
- Autoimmune diseases like insulin-dependent (juvenile) diabetes, multiple sclerosis, rheumatoid arthritis and psoriasis

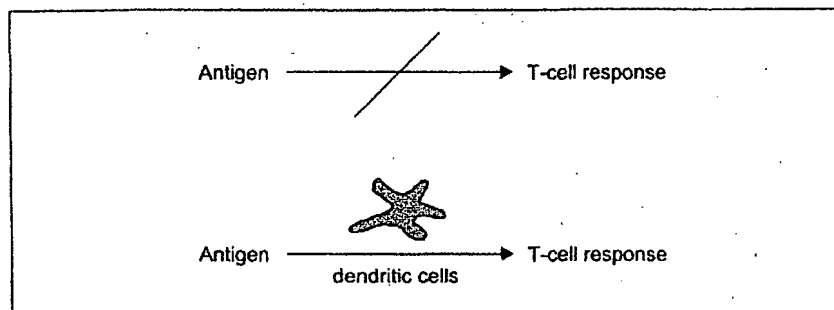


Fig. 1. A key function of dendritic cells. Antigens within tumors, transplants and infectious agents need to be presented by DCs to become immunogens, i.e., to make T cells begin to grow and exhibit their helper and killer functions.

to other players in the immune system such as B cells, T cells and macrophages. In reality, DCs are quite abundant for the job they have to do, namely, to initiate immune responses from antigen-specific T cells. In immune system organs like lymph nodes, DCs form an extensive network throughout the T cell-rich regions and physically outnumber any given antigen-reactive T cell by at least 100 to 1. The DC field was also held back because many thought that the cells were no different from macrophages, thus keeping investigators from working on the active DCs. In reality, DCs were identified on the basis of profound differences from macrophages, and their many distinct properties and functions were only uncovered by separating DCs from macrophages.

The best-studied function of DCs is to convert antigens into immunogens for T cells. The antigen receptors on T cells do not focus on intact proteins in microbes and tumors, but instead recognize fragmented or processed proteins, that is, peptides. The processing of protein antigens into peptides occurs within cells, and then the peptides are

displayed or presented at the cell surface affixed to products of the major histocompatibility complex (MHC). The ensuing interaction between a T-cell receptor (TCR) and its specific MHC-peptide complex allows a T cell to detect peptides formed within cells in transplants, tumors, sites of infection and self tissues attacked during autoimmune disease (Table 1). "Antigens" refers to specific substances recognized by the immune system, while "immunogens" refers to antigens that effectively induce responses either by themselves or together with enhancing materials called "adjuvants." For T cells in particular, antigens and immunogens are not one and the same (Fig. 1). Even preprocessed peptides and MHC-peptide complexes are weak immunogens. This was evident early on in the work of Peter Medawar, the great scientist who discovered the immune basis of transplantation. He spent many years trying to purify functioning transplantation antigens. These efforts were to little avail.

What was not known in Medawar's time is that transplantation antigens

(later shown to be MHC-peptide complexes) become immunogenic when presented by DCs.<sup>4</sup> In other words, transplantation antigens when presented on many cell types are weak immunogens, but on DCs they become powerful inducers of immunity.<sup>4</sup> The same is true of peptides that become much more immunogenic when presented on DCs. DCs activate T cells by getting them to divide and express their helper and killer functions. Then the activated T cells interact with other antigen-presenting cells to eliminate the antigen in question. DCs are also called "nature's adjuvant," because prior adjuvants were artificial substances used to enhance immunity. The DC advantage entails a myriad of functions, some of which will be considered below.

### Potency of dendritic cells in initiating immunity in tissue culture

What are some specific features of DCs that warrant attention? The first is their potency. Very small numbers of DCs are sufficient to trigger strong T-cell responses in test tubes. Immune assays are generally carried out with impure antigen-presenting cells, applied at a dose of one presenting cell for every T cell, the latter often preactivated. In contrast, roughly one DC per 30-100 T cells is more than sufficient to induce optimal responses, including responses by resting T cells. A single DC can simultaneously activate 10-20 T cells nestled within its sheet-like processes. Therefore, DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity.

It has always been clear that the accessory function of DCs did not depend exclusively on their capacity to process antigens to form MHC-peptide complexes. This is because the stimuli that were used to define the potency and immune-activating role of DCs did not require that the DCs process an applied antigen. Such stimuli included major transplantation anti-

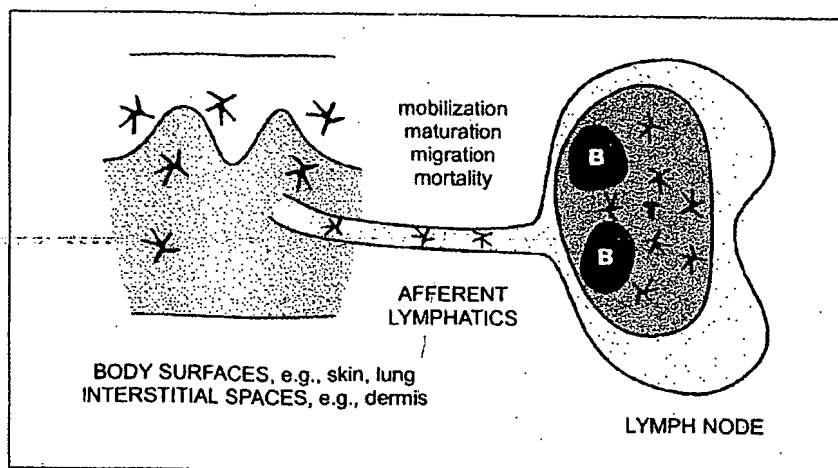
T-cell antibodies and superantigens. Furthermore, once resting T cells were activated by DCs, the T cells responded vigorously to antigens presented by other cell types, showing that the latter were not deficient in forming ligands for the antigen receptor on T cells, but instead lacked accessory properties.

The word "accessory" has since been replaced by the terms "professional" and "co-stimulatory," but the basic concept is unchanged by shifting terminology. T cells need stimuli other than their specific trigger or ligand (MHC-peptide complexes) to begin to grow and function, for example, to produce the interleukins and killer molecules mentioned above. DCs are potent in providing the needed accessory or co-stimulatory functions. For example, DCs produce an adhesion molecule called DC-SIGN that binds to a target on resting T cells called ICAM-3,<sup>5</sup> and DCs express very high levels of a stimulatory molecule called CD86 that binds to CD28 on resting T cells.<sup>6</sup> These are but two examples of the specialized activities of DCs. These cells do not operate as a single magic bullet.

### Position of dendritic cells *in vivo*

Another special feature of DCs is their location and movement in the body. As criteria were developed to identify DCs, it became feasible to go back into the animal and patient to look for the corresponding cells in different tissues. DCs are stationed at surfaces where antigens gain access to the body (Fig. 2, left). The skin and the airway have been the best studied. DCs are found in afferent lymphatic vessels, special channels that allow cells to move from peripheral tissues to lymphoid organs, primarily the T-cell areas (Fig. 2, middle and right). This migration is most readily observed in models of skin transplantation and contact allergy, which are the two most powerful immune responses known.

DC migration is likely to be very important. The body's pool of T cells primarily traffics through the T-cell areas of lymph nodes, rather than



**Fig. 2.** Distribution of dendritic cells *in situ*. DCs at body surfaces and in solid organs can pick up antigens, move to the lymphoid tissues to find antigen-specific T cells and initiate immunity. Molecular mechanisms are being uncovered that govern the mobilization, maturation, migration and mortality of these DCs. In the lymph node, T lymphocytes are selected for expansion and differentiation into helper and killer T cells. The activated T cells then leave the lymph node to return to the body surface or peripheral organ to eliminate the antigen.

through tissues where antigens are usually deposited. So when DCs capture antigens in the skin, airway or another peripheral tissue, their migration to the T-cell areas gives them a chance to select the corresponding rare specific T cells from the assembled repertoire (Fig. 2). The selected T cells then increase in numbers (clonal expansion) and function, enabling the specific immune response to begin. The initial frequency of T cells that recognize an antigen is very small. Only one in 10,000–100,000 of T cells in the repertoire responds to a specific MHC-peptide complex. Therefore, it is so precise and efficient for DCs to be able to pick up an antigen in the periphery and then initiate the immune response from rare T-cell clones in lymphoid organs.

The events that make up the life history of DCs (Figs. 2 and 3) are now being unraveled in molecular terms. For example, scientists are figuring out how to expand antigen-capturing precursors to DCs using Il13 ligand and granulocyte colony-stimulating factor (G-CSF). Key players for the mobilization of DCs from the periphery to lymph nodes are the multidrug resistance receptors, usually studied for their capacity to mediate resistance to chemotherapeutic agents rather than

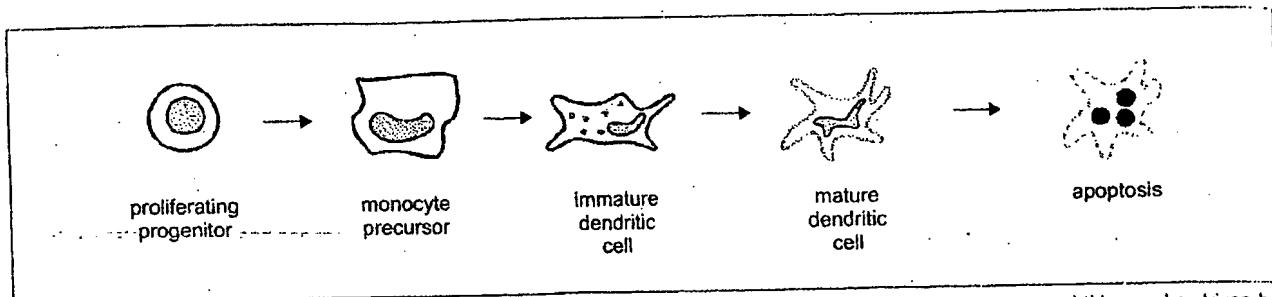
movement of DCs. Migration of DCs is controlled by chemokines produced in the lymphatic vessels and lymphoid organs (Fig. 2). These act on DC chemokine receptors to orchestrate their movement to the T-cell areas. Then within the lymphoid tissue, several members of the tumor necrosis factor (TNF) and TNF-receptor families, such as TRANCE and CD40 ligand, trigger DC production of cytokines like interleukin-12. The TNF family also maintains DC viability. Otherwise the cells die within a day or two. Each of these components of DC function provides targets for manipulating immunity.

### Priming of T-cell immunity via dendritic cells

#### Animal studies

During the early research on DCs, several labs administered antigens to experimental animals and then tried to identify the cells that had captured the antigens in a form that was immunogenic. Regardless of the route of antigen administration (blood, muscle, skin, intestine and airway), DCs were the major reservoir of immunogen.





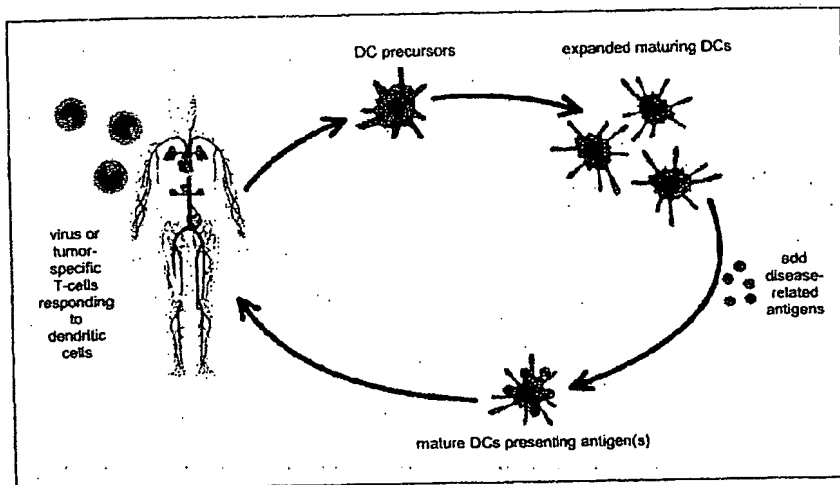
**Fig. 3.** The life history of dendritic cells. DCs arise from proliferating progenitors, primarily in the bone marrow, and this can be driven by cytokines like flt-3 ligand and G-CSF. Precursors are formed, such as the monocytes in blood, which then give rise to immature DCs. The immature DCs are capable of producing large amounts of antigen-presenting MHC products and capturing antigens. Multidrug resistance receptors are newly recognized players in the mobilization of immature DCs. DCs mature in response to various stimuli such as infection and inflammation, and migrate under the influence of chemokines to the lymphoid tissues. There the DCs die within a day unless their life-span is prolonged by TNF-family molecules expressed by the activated T cells.

Next, DCs were used as nature's adjuvant to immunize animals. The DCs were taken from mice or rats, exposed to antigens *ex vivo* and injected back into immunologically naive recipients. The animals became immunized to the antigens that had been captured by the DCs, and the immunization took place in the lymph nodes draining the site of DC injection. Genetic proof was provided that the DCs were priming the animal directly and not simply handing off their antigen to other cells.<sup>7,8</sup>

DC-based immunization is really very different from all prior attempts at cell therapy. Immunology has had extensive experience with "passive immunization," whereby a recipient is given large numbers of cells that are activated prior to injection. It is hard to produce such large numbers of cells, and their lifespan, diversity and efficacy are all finite. In contrast, when relatively small numbers of antigen-charged DCs are used to induce immunity, this produces "active immunization." Now the animals (and patients, see below) can make their own diverse and longer-lasting immune response to the antigen-bearing DCs.

### Human studies

The above experiments made it clear that DCs, pulsed *ex vivo* with antigens, actively immunized animals and raised the exciting possibility that scientists would be able to induce resis-



**Fig. 4.** The use of dendritic cells as adjuvants for enhancing immunity to tumors and infectious agents in humans. This new form of immune therapy begins with the isolation of DC precursors from the patient, usually from blood. The precursors develop *ex vivo* (in relatively simple tissue culture systems) into large numbers of more mature DCs. During this time, the DCs are charged with antigens from the tumor or infection. Then the DCs are reinfused to elicit immunity and thereby resistance to the disease.

tance to tumors, infections and transplants in patients. For example, could one expose patients' DCs *ex vivo* to antigens in their tumors and then reinfuse the antigen-bearing DCs to elicit tumor-specific immunity (Fig. 4)? This approach is actually not terribly complicated, but one first had to overcome a major obstacle and learn to generate large numbers of DCs. These techniques became available in the 1990s. They have energized the field and, accordingly, clinical trials for the immunization of humans against cancer have begun on most continents.

It is evident that DCs can serve as adjuvants for humans, converting antigens into immunogens.<sup>9,10</sup> Even in advanced cancer, immune responses already have been observed that are similar to or better than immune responses obtained with other approaches. However, this approach is still in its preliminary stages, since a good deal of science remains to be developed. On the one hand, there are critical unknowns in terms of overall DC biology. Many of the clinical studies to date, for example, have overlooked key features that could improve DC function, such as the need for DCs

to be sufficiently mature (see below) to be effective *in vivo*. Also, DC biology has to be placed in the context of specific tumors and pathogens and patients for DC-based therapies to be optimized.

To summarize and further illustrate the role of DCs in the context of human disease (Table I), consider the need to harness T cells to resist tumors and chronic infections. Protein antigens often are known for a tumor-like melanoma, or for a virus like HIV-1 whose genetic sequence has been available for more than 15 years. However, this knowledge about antigens from melanoma and HIV-1 antigens remains to be converted into methods that provide better immunogens either for immune therapy of melanoma or for the design of HIV-1 vaccines. This is because some important facts of immunological life are being overlooked. When antigens are injected, they also need to gain access to the right DCs to become immunogens (Fig. 1).

### Delivering antigens to dendritic cells

Broadly speaking, a central goal is to learn how to deliver or "target" antigens to DCs and simultaneously to differentiate or "mature" the cells to their most potent state. These two challenges, antigen targeting and DC maturation, prove to be intertwined.

Targeting means that the antigen should be in a form that the DCs can recognize. Without such recognition, the uptake and subsequent processing of antigen to form MHC-peptide complexes is suboptimal. DCs have a number of special mechanisms for capturing antigens and converting these into MHC-peptide complexes (Table II). For example, DCs have a receptor called DEC-205 whose binding partners or ligands are still unknown. Nonetheless, it is clear that DEC-205 greatly increases the capacity of DCs to form MHC-peptide complexes.<sup>11</sup> DCs also carry out a fascinating process called "cross-presentation." DCs can take up dying cells and effi-

TABLE II: DENDRITIC CELL SPECIALIZATION TO INCREASE MHC-PEPTIDE COMPLEX FORMATION

- Receptors for antigen uptake, e.g., DEC-205
- Processing of dying cells, nonreplicating microbes and immune complexes onto MHC class I ("cross-presentation")
- Regulation of antigen processing by maturation stimuli
- Clustering of T-cell receptor ligands with co-stimulators like CD86

ciently extract peptides from them, so antigens "cross" from the dying cell to the DC. The discoverers of this phenomenon called it "resurrecting the dead."<sup>12</sup> Cross-presentation allows DCs to efficiently form MHC-peptide complexes from dead cells in tumors, transplants and tissues under autoimmune attack.

Special uptake and processing mechanisms allow DCs to tailor a protein antigen, as well as the proteins in a complex microbe or tumor cell, into peptides that bind to an individual's MHC products. The latter are exceptionally polymorphic, differing genetically from one individual to another. As a result, the relevant immunizing peptides differ from one individual to another. One reason why peptides are not ideal immunogens is that they must be individualized. DCs, in contrast, can capture antigens with high efficiency and likewise extract peptides that are relevant for any individual.

A second DC advantage is that these cells have the many required accessory or co-stimulatory properties for converting the selected peptides ("antigens") into effective immunogens. A third DC advantage is that these cells position themselves in a way that leads to the identification of rare antigen-reactive T lymphocytes *in vivo* (Fig. 2). DCs thus overcome many of the difficult obstacles in initiating immunity.

In order for an antigen to be a strong immunogen, one needs to provide a stimulus for the final differentiation or maturation of the DCs (Fig. 3). Most DCs in the body are in an immature state and lack many features that lead to a strong T-cell response.

Immature DCs, for example, lack the CD86 and CD40 molecules that greatly boost the DC-T cell interaction. Immature DCs also lack a chemokine receptor called CCR7 that seems very important for proper migration and homing to lymph nodes to start immunity. For cancer immunology, it is unlikely that tumors provide maturation stimuli. Tumors may even block DC maturation induced by other stimuli. Therefore it is important to learn how to deliver tumor cells to DCs and bypass the normal obstacles to effective antitumor immunity.

Surprising recent evidence actually links DC maturation to the efficient formation of MHC-peptide complexes or TCR ligands (Table II). Immature DCs take up antigens, but they do not make abundant MHC-peptide complexes until they receive a maturation stimulus.<sup>13,14</sup> Maturation also up-regulates CD86 co-stimulators, but the CD86 actually travels together with the TCR ligands to the surface of the DCs. At the DC surface, the MHC molecules and CD86 remain clustered with each other, keeping the machinery for T-cell activation juxtaposed. This phenomenon will help explain the potency of DCs, because TCR ligands and co-stimulators are displayed together on the cell surface and in high levels.

### Control points beyond antigen targeting and maturation of DCs

Research on DCs is moving more vigorously, because the cells are more readily available and because their role in the immune system is considered essential. Nonetheless, researchers in this field are just beginning to find ways to manipulate DCs *in situ*. Putting together an antigen that targets

to DCs with a stimulus for DC maturation will be a major step in improving the conversion of antigens into immunogens, as in immune-based therapies against tumors and infectious agents.

Additional challenges and questions are evident:

- How can DC numbers be increased *in situ* and how can active DCs be mobilized to a cancer or site of chronic infection?
- Can DCs induce strong immune memory to make vaccination long lasting and effective (we have only been reviewing the role of DCs in the initiation of immunity)?
- Can DCs change the quality of the immune response? "Quality" refers to recent evidence for different types of DCs, especially a subset that induces Th1-type T cells for resistance to infectious agents and strong memory.
- Is it possible to move beyond DC-based immunization experiments and use DCs to either regulate or tolerize the immune system, as frequently required in transplantation and autoimmune diseases?
- Can DCs influence elements of the immune system other than T cells; for example, B cells and the innate defenses provided by natural killer (NK) and NK-T cells?

The answer to all these questions is a preliminary "yes." As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

### Dendritic cells and better control of disease

DCs provide important avenues for the investigation of human disease. Many labs are exploiting DCs to identify antigens relevant for immunity against human pathogens. In these experiments, one introduces complex but clinically important antigens to DCs and then identifies which components are best presented to the immune system. We have recently used this approach to identify previously un-

known immune responses to the Epstein-Barr virus,<sup>15</sup> a virus we all carry that has the potential to cause cancer like Hodgkin's lymphoma. Other laboratories have been using DCs to identify new antigens in other infectious agents, in transplants and in cancers like melanoma.

Investigators are also manipulating DCs *ex vivo* and then reinfusing the cells to identify conditions leading to strong immunity in patients (Fig. 4). In particular, DC-mediated active immunization against cancer is being vigorously pursued, as mentioned above. Instead of manipulating DCs *ex vivo*, a more desirable goal would be able to alter DCs directly *in situ*. Some approaches are under way. An example is the injection of cytokines like flt3 ligand and G-CSF to mobilize various precursor populations of DCs. One should also develop methods to control DC mobilization, migration and maturation. In sum, DCs are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

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## Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor

(heterodimeric lymphokine/T-cell growth factor/lymphokine-activated killer cells/coordinate gene regulation/interleukin-12)

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**ABSTRACT** Cytotoxic lymphocyte maturation factor (CLMF) is a disulfide-bonded heterodimeric lymphokine that (i) acts as a growth factor for activated T cells independent of interleukin 2 and (ii) synergizes with suboptimal concentrations of interleukin 2 to induce lymphokine-activated killer cells. We now report the cloning and expression of both human CLMF subunit cDNAs from a lymphoblastoid B-cell line, NC-37. The two subunits represent two distinct and unrelated gene products whose mRNAs are coordinately induced upon activation of NC-37 cells. Coexpression of the two subunit cDNAs in COS cells is necessary for the secretion of biologically active CLMF; COS cells transfected with either subunit cDNA alone do not secrete bioactive CLMF. Recombinant CLMF expressed in mammalian cells displays biologic activities essentially identical to natural CLMF, and its activities can be neutralized by monoclonal antibodies prepared against natural CLMF. Since this heterodimeric protein displays the properties of an interleukin, we propose that CLMF be given the designation interleukin 12.

The molecular cloning and expression of recombinant cytokines has made possible both significant advances in our understanding of the molecular basis of immune responses and the development of new approaches to the treatment of disease states. As an example, recombinant interleukin 2 (recombinant IL-2) has been shown to be capable of causing regression of established tumors in both experimental animals (1) and in man (2); however, its clinical use has been associated with significant toxicity (2). One potential approach to improving the therapeutic utility of recombinant cytokines is to use them in combination (3, 4). With this concept in mind, we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo*. This led to the identification of a factor, designated cytotoxic lymphocyte maturation factor (CLMF), that synergized with recombinant IL-2 to facilitate the generation of both cytolytic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells *in vitro* (5, 6). CLMF was subsequently purified to homogeneity from a human lymphoblastoid B-cell line (NC-37) and was shown to be a 75-kDa disulfide-bonded heterodimer composed of two subunits with molecular masses of 40 kDa and 35 kDa (7).<sup>†</sup> We now report the molecular cloning and expression of CLMF.

## MATERIALS AND METHODS

**cDNA Cloning.** A subline of NC-37 cells selected for its ability to produce high levels of CLMF (7), NC-37.98, was induced with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 for 16 hr. Poly(A)<sup>+</sup> RNA was isolated, and random hexamer-primed cDNA libraries were established in phage  $\lambda$ gt10 by standard procedures. Mixed-primer polymerase chain reaction (PCR) using controlled ramp times (8) was performed as follows. PCR primers contained all possible codons and were 14 or 15 nucleotides long (Fig. 1) with a 5' extension of 9 nucleotides containing an *Eco*RI site for subcloning. Degeneracies varied from 1 in 32 to 1 in 4096; 0.5–4 pmol per permutation of forward and reverse primer was used in a 50- to 100- $\mu$ l PCR mixture with 40 ng of cDNA made from NC-37.98 cells that had been activated by culture with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml for 16 hr (40-kDa subunit) or with 3  $\mu$ g of human genomic DNA (35-kDa subunit). PCR cycling parameters were as follows. Initial denaturation was at 95°C for 7 min. Low-stringency annealing was performed by cooling to 37°C over 2 min, incubating 2 min at 37°C, heating to 72°C over 2.5 min, extending at 72°C for 1.5 min, heating to 95°C over 1 min, and denaturing at 95°C for 1 min. This cycle was repeated once. Thirty standard cycles (40-kDa subunit) or 40 standard cycles (35-kDa subunit) were performed as follows: 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. "Amplicons" of the expected size were gel-purified, subcloned, and sequenced. The 40-kDa subunit cDNAs were isolated by hybridizing the 54-mer amplicon in 5 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 20% formamide at 37°C overnight. Filters were washed in 2 $\times$  SSC at 42°C for 30 min and exposed to x-ray film. The 35-kDa subunit cDNAs were isolated by hybridizing the 51-mer amplicon in 5 $\times$  SSC/20% formamide at 37°C overnight. The filters were washed in 2 $\times$  SSC at 40°C for 30 min and exposed to x-ray film. Positive clones were plaque-purified, their inserts were subcloned into the pBluescript plasmid, and their sequences were determined by using Sequenase.

**Expression.** cDNAs were separately engineered for expression in vectors containing the simian virus 40 early promoter essentially as described (9). COS cells were transfected with both CLMF subunit expression plasmids mixed together or

Abbreviations: CLMF, cytotoxic lymphocyte maturation factor; rCLMF and nCLMF, recombinant and natural CLMFs; CTL, cytolytic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; n, natural; PCR, polymerase chain reaction.

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<sup>‡</sup>The cDNA sequences reported in this paper have been deposited in the Genbank data base [accession nos. M38443 (35-kDa CLMF subunit) and M38444 (40-kDa CLMF subunit)].

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1  MCPARSLLLV ATLVLDDHLS LARNLPVATP DPGMFPC^LHH SQNLLRAVSN
51  MLQKARQ^TLE FYPCTSEEID HEDITKDKTS TVEAC^LPLEL TKNESC^LN^SR
101  ETSFITNGSC LASRKTSFMM ALCLSSIIYED LKMYQVEFKT MNAKLLMDPK
151  RQIFLDQNML AVIDELMQAL NFNSETVPQK SSLEEPDFYK TKIKLCIL^LH
201  AFRIRAVTID RVTSYLNAS

1  MCHQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVVLTC
51  DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVL^SHS
101  LLLLHKKEDG IWSTDILK^DQ KEPKNKTF^LR CEAKNYSGRF TCWWLT^TIST
151  DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSAC^PF
201  AAESLPIEV MDAVHKLY ENYTSSFFIR DIIKPDPPKN LQLKPLKNSR
251  QVEVSWEYPD TWSTPHSYFS LTFVCVQVGK SKREKKDRVF TDKTSATV^IC
301  RKNASISVRA QDRYSSSSWS EWASVPCS

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FIG. 1. Amino acid sequences of the 35-kDa (Upper) and 40-kDa (Lower) CLMF subunits as deduced from the respective cDNAs and shown in single-letter code. Signal peptides are overlined, cysteine residues are marked by a caret, and N-linked glycosylation sites (NXS, NXT, where X is another amino acid) are underlined. The peptide sequences used to generate PCR probes are overlined with arrows indicating the direction of amplification.

with each one separately by the DEAE-dextran method. Twenty-four hours after transfection, the serum-containing medium was replaced with medium containing 1% Nutridoma-SP (Boehringer Mannheim), and supernatant fluids were collected from the cultures after 40 hr. These fluids were stored at 4°C until tested in the bioassays.

**General Methods.** Standard molecular biology procedures were used as described (10). CLMF bioassays were performed as detailed (7).

**Computer Searches.** The National Biomedical Research Foundation protein data base (Release 26.0) as well as the Genbank and European Molecular Biology Laboratory databases (Releases 65.0 and 24.0, respectively) were searched for sequences homologous to CLMF cDNAs. The two subunit sequences were compared to each other using the ALIGN program (mutation data matrix, break penalty of 6; see ref. 11).

## RESULTS

Partial N-terminal amino acid sequences of the two CLMF subunits (7) were used to generate completely defined 51- to 54-base-pair (bp)-long oligonucleotide probes by means of mixed primer PCR. These probes were used to screen cDNA libraries made from RNA from activated NC-37.98 cells, and cDNAs encoding the two subunits were isolated and characterized. Both cDNAs encode secreted proteins with a classical hydrophobic N-terminal signal peptide immediately followed by the N terminus of the mature protein as determined by protein sequencing (7). Two independent cDNA clones for the 40-kDa subunit were shown to be identical. Both encode the mature 40-kDa subunit that is composed of 306 amino acids (calculated  $M_r = 34,699$ ) and contains 10 cysteine residues and four potential N-linked glycosylation sites (Fig. 1). Two of these sites are within isolated tryptic peptides derived from the purified 40-kDa CLMF subunit protein. Amino acid sequence analysis has shown that Asn-

222 is glycosylated, whereas Asn-125 is not (Fig. 1; F. Podlaski, personal communication). The mature 35-kDa subunit is composed of 197 amino acids (calculated  $M_r = 22,513$ ), with 7 cysteine residues and three potential N-linked glycosylation sites (Fig. 1). When purified CLMF is reduced with 2-mercaptoethanol and analyzed by SDS/PAGE, the 35-kDa subunit appears to be heterogeneous, suggesting that it may be heavily glycosylated (7). Two variants of 35-kDa subunit-encoding cDNAs were isolated. The first type had the sequence shown in Fig. 1. Additional isolates contained what is probably an allelic variation, replacing Thr-213 with a methionine residue.

Computer searches of sequence databases showed that the amino acid sequences of the two subunits are not related to any known protein. The subunit sequences are also not related to each other, since a comparison using the ALIGN program (11) gave a score of 1.27; only scores >3 are considered to indicate significant evolutionary relationship (12). The genes encoding the subunits appear to be unique, since low- and high-stringency hybridizations of genomic blots revealed identical banding patterns (data not shown). RNA blots showed the size of the 40-kDa subunit mRNA to be 2.4 kb, whereas the 35-kDa subunit was encoded by a 1.4-kb transcript (Fig. 2). Expression of the two mRNAs encoding the subunits was coordinately regulated upon induction (Fig. 2). When NC-37.98 cells were activated with PMA and calcium ionophore for 72 hr, mRNA encoding each of the CLMF subunits was minimally detectable at 6 hr after the beginning of induction but was readily detected at 24 hr and continued to accumulate until maximal levels were reached at 72 hr (normalized to GAPDH mRNA levels; see the legend to Fig. 2). In contrast, the mRNA for IL-2 in activated NC-37.98 cells was already at high levels at 6 hr and subsequently decreased, whereas the mRNAs for the low-affinity IL-2 receptor (p55) followed the induction pattern seen for the CLMF subunits. Scanning of RNA blots also revealed that steady-state mRNA levels for the 40-kDa

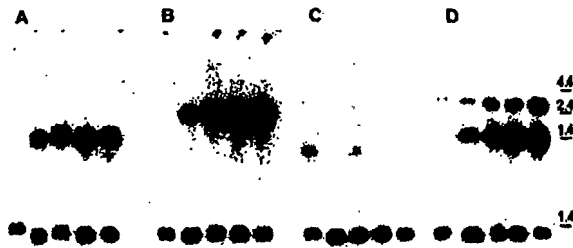


FIG. 2. RNA blots showing the coordinate induction of the 35-kDa (A) and 40-kDa (B) CLMF subunit mRNAs and IL-2 mRNA (C) and its p55 receptor mRNA (D). Poly(A)<sup>+</sup> RNA (5  $\mu$ g) from NC-37.98 cells activated with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml were loaded in each lane. Lanes from left to right in each panel show RNAs isolated 6, 24, 30, 48, and 72 hr after induction, respectively. (Upper) Four-day exposures. (Lower) Two-hour exposure of the same blots after stripping and rehybridization with a GAPDH probe. Marker sizes are in kb (BRL RNA ladder).

CLMF subunit were severalfold higher than those for the 35-kDa subunit expressed by the same cells. This finding parallels the observation that activated NC-37 cells secrete excess free 40-kDa subunit protein (7). The 3' untranslated sequences of both CLMF subunit mRNAs contain several copies of the octamer motif TTATTAT (data not shown). This sequence is present in other transiently expressed mRNAs and is involved in regulating mRNA stability (13).

Coexpression of the 40-kDa and 35-kDa CLMF subunit cDNAs in COS cells was required to generate secreted biologically active CLMF (Table 1 and Fig. 3). COS cells transfected with cDNA encoding either the 40-kDa subunit alone or the 35-kDa subunit alone did not secrete biologically active CLMF (Table 1). Mixing media conditioned by COS

cells that had been separately transfected with one or the other of the two CLMF subunit cDNAs also did not give rise to bioactive CLMF (Table 1).

Two types of assays were used to compare rCLMF and nCLMF. The first assay measures the proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes, whereas the second assay evaluates the synergy between CLMF and suboptimal concentrations of IL-2 in the generation of LAK cells in hydrocortisone-containing cultures (7). The data in Fig. 3 show that rCLMF as expressed in COS cells and nCLMF as purified from NC-37 cells are essentially identical. Dose-response curves for rCLMF and nCLMF were superimposable in each of the two assays, and rCLMF was neutralized by a monoclonal antibody raised against nCLMF. Conditioned media from cultures of mock-transfected COS cells displayed no activity in these assays (Table 1 and data not shown).

## DISCUSSION

In a previous report (7), we described the purification of a heterodimeric cytokine, CLMF, that synergized with low amounts of IL-2 to cause the generation of LAK cells in the presence of hydrocortisone and stimulated the proliferation of activated T cells independent of IL-2. In the present report, we have used the N-terminal amino acid sequence information previously obtained to clone the two subunit cDNAs of CLMF. Protein purification of NC-37 cell line-derived CLMF had shown that the protein was composed of two disulfide-bonded subunits with different N-terminal amino acid sequences (7). However, it was not clear from our previous results whether the two subunits were processed from one common gene product and whether proteolytic posttranslational processing other than signal peptide cleavage was occurring. The molecular cloning and sequencing of

Table 1. Coexpression of both CLMF subunit cDNAs is required for secretion of biologically active CLMF by COS cells

Addition	Conc., units/ml	Dilution	[ <sup>3</sup> H]Thymidine incorporated by PHA-activated lymphoblasts, mean cpm $\pm$ 1 SEM
Cytokine*			
None	—		11,744 $\pm$ 514
nCLMF	200		68,848 $\pm$ 878
nCLMF	40		48,827 $\pm$ 605
nCLMF	8		26,828 $\pm$ 594
nCLMF	1.6		17,941 $\pm$ 196
Culture fluid from COS cells transfected with			
A. 35-kDa CLMF subunit cDNA		1:20	11,912 $\pm$ 660
		1:100	10,876 $\pm$ 232
B. 40-kDa CLMF subunit cDNA		1:20	11,699 $\pm$ 931
		1:100	11,666 $\pm$ 469
C. 35-kDa + 40-kDa CLMF subunit cDNAs		1:20	58,615 $\pm$ 587
		1:100	38,361 $\pm$ 828
1:1 mix of culture fluids A and B		1:10 <sup>†</sup>	11,544 $\pm$ 483
		1:50	10,503 $\pm$ 259
CM from mock-transfected control <sup>‡</sup>		1:20	11,503 $\pm$ 286
		1:100	10,751 $\pm$ 303

PHA-activated lymphoblasts were prepared from human peripheral blood mononuclear cells as described (7). Lymphoblast proliferation was measured in a 48-hr assay (7) in which  $2 \times 10^4$  lymphoblasts were incubated in 100- $\mu$ l cultures containing the indicated amounts of natural CLMF (nCLMF) or COS cell culture fluids. [<sup>3</sup>H]Thymidine was added to each culture 18 hr prior to harvest. Conc., concentration.

\*nCLMF is purified NC-37-derived CLMF.

<sup>†</sup>1:10 dilution of the 1:1 mixture of culture fluids A and B was equivalent to a 1:20 final dilution of each of the individual culture fluids.

<sup>‡</sup>Conditioned medium (CM) from cultures of mock transfected COS cells.

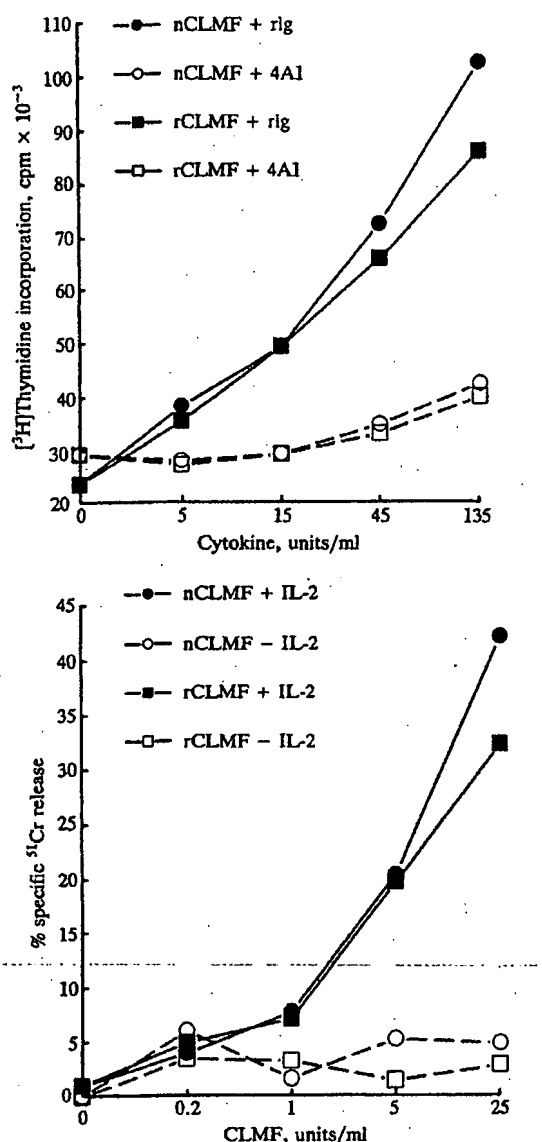


FIG. 3. Comparison of biologic activities of nCLMF (circles) and recombinant CLMF (rCLMF, squares). nCLMF was purified from NC-37 cell-conditioned media; rCLMF was purified from conditioned media from cultures of COS cells transfected with cDNAs encoding the 40-kDa and 35-kDa human CLMF subunits. (Upper) T-cell growth factor assay. The ability of CLMF to stimulate the proliferation of human PHA-activated lymphoblasts in 48-hr cultures was assayed as described (7). CLMF preparations were mixed with neutralizing rat monoclonal anti-human CLMF antibody 4A1 (ref. 7; open symbols) or with normal rat IgG (Sigma; rlg, closed symbols) at a final concentration of 20  $\mu$ g of IgG/ml and were incubated for 30 min at 37°C prior to addition of PHA blasts. All values are means of triplicate determinations. (Lower) LAK cell induction assay. The ability of CLMF, alone or in combination with recombinant IL-2, to induce the generation of LAK cells in 4-day cultures was assessed as described (7). Low-density peripheral blood lymphocytes were incubated in the presence of various amounts of nCLMF or rCLMF with (closed symbols) or without (open symbols) recombinant IL-2 at 7.5 units/ml. Units of CLMF activity were based on previous titrations in the T-cell growth factor assay. Hydrocortisone sodium succinate (Sigma) was included at a concentration of 0.1 mM to minimize triggering of endogenous cytokine cascades. Lysis of <sup>51</sup>Cr-labeled Daudi cells was assessed at an effector/target ratio of 5:1. The data shown represent the means of quadruplicate determinations. The spontaneous <sup>51</sup>Cr release was 20%.

the corresponding cDNAs now has demonstrated that there is no common precursor for the two CLMF subunits; rather, they are encoded by completely different genes. The predicted and actual amino acid composition for the two subunits are strikingly similar; differences in predicted versus actual molecular weights are accounted for by glycosylation (F. Podlaski, personal communication). Thus, the only major posttranslational proteolytic event that appears to take place in the maturation of the CLMF subunits is signal peptide cleavage.

The kinetics of expression of the individual CLMF subunit mRNAs were examined and compared to the expression of mRNAs for IL-2 and the IL-2 receptor p55. Previously it had been observed that NC-37 cells, like certain murine (14) and marmoset (15) B-cell lines, secreted IL-2 when activated (M.K.G., unpublished results). RNA blots demonstrated that upon activation of NC-37 cells, both CLMF subunit mRNAs were coordinately induced with kinetics similar to the IL-2 receptor (p55) mRNAs. On the other hand, IL-2 mRNA levels peaked much earlier. Similar differences in induction kinetics were also seen at the level of IL-2 and CLMF bioactivity secreted from NC-37 cells (M.K.G., unpublished data). These kinetic differences are consistent with our previous observation that in a cytolytic lymphocyte response, IL-2 appears to act earlier than CLMF (5).

Transfection studies with COS cells established that only coexpression of both subunit cDNAs gives rise to secreted bioactive CLMF. Thus, it appears that the two proteins have to interact within the endoplasmic reticulum to assemble properly into bioactive secreted CLMF. By comparing the activity of rCLMF to that of nCLMF in the T-cell growth factor and LAK cell induction assays (Fig. 3) and assuming that the specific activity of rCLMF is similar to that of nCLMF ( $8 \times 10^7$  units/mg (7)), we estimate that the amount of rCLMF heterodimer produced in these experiments was 5–50 ng/ml. The finding that COS cells, which are fibroblast-like cells, are able to assemble correctly the two CLMF subunits to form bioactive CLMF indicates that this secretion and processing pattern is not limited to cells of the lymphoid lineage.

Western blot analysis using an anti-CLMF antibody specific for the 40-kDa subunit has allowed confirmation that (i) COS cells transfected with both CLMF subunit cDNAs secrete CLMF with the expected heterodimeric structure and (ii) COS cells transfected with the 40-kDa subunit cDNA alone secrete that subunit (F. Podlaski, personal communication). Since no bioactivity was detected in media conditioned by COS cells transfected with only the 40-kDa subunit, that subunit by itself appears either to have a much reduced specific activity compared with heterodimeric CLMF or to be completely inactive.

Because of the lack of a high-affinity antibody specific for the 35-kDa subunit, we have not yet been able to determine definitively whether COS cells transfected with only the 35-kDa subunit cDNA secrete that subunit. Since no bioactivity was detected in the media, secretion of a bioactive 35-kDa subunit by itself could be very inefficient; alternatively, similar to the 40-kDa subunit, the protein could be much less active or inactive altogether. Intracellular 35-kDa protein in the absence of the other subunit could be inherently unstable; there is precedence for this phenomenon, since it has been reported that 90% of the  $\beta$  chains of lutropin (LH), when expressed in the absence of  $\alpha$  chains, are retained in the endoplasmic reticulum and are slowly degraded (16). Simple mixing of media conditioned by COS cells transfected separately with either one of the two CLMF subunit cDNAs did not yield bioactive CLMF. One possible explanation would be that the cells do not secrete the 35-kDa CLMF subunit by itself. More likely, our experimental conditions did not allow proper heterodimer formation. One would expect that only

carefully controlled renaturation and oxidation conditions would allow the disulfide bond formation required for generation of bioactive CLMF.

Normal human peripheral blood lymphocytes under the appropriate induction conditions produce both CLMF subunit mRNAs and secrete the active protein (N.N. and M.K.G., unpublished data). There is some evidence suggesting that CLMF is produced predominantly by B cells. In preliminary experiments, B-cell mitogens have appeared to be more effective than T-cell mitogens in eliciting CLMF production from peripheral blood lymphocytes (M.K.G., unpublished results). When screening human cell lines for their ability to produce CLMF activity (7), we observed that four of eight B-cell lines tested produced CLMF after activation with PMA and calcium ionophore, whereas none of five T-cell lines produced CLMF. Nevertheless, three of these T-cell lines secreted large amounts of IL-2 and tumor necrosis factor activity after activation (M.K.G., unpublished results). Likewise, natural killer cell stimulatory factor (NKSF), a heterodimeric cytokine similar or identical to CLMF, was isolated from RPMI 8866 lymphoblastoid B cells (17). A recent report (18) has indicated that B lymphocytes can secrete a cytokine(s) distinct from IL-2 that facilitates virus-specific cytolytic T-lymphocyte responses. It is possible that CLMF may have been the cytokine active in those studies. Thus, although B lymphocytes have not traditionally been viewed as cytokine-producing helper cells, it is conceivable that CLMF production constitutes a novel mechanism whereby B lymphocytes contribute to the amplification of T-lymphocyte responses. In addition to the biologic activities described in this report, CLMF by itself has been shown (i) to activate NK cells in an 18–22 hr assay, (ii) to facilitate the generation of specific allogeneic CTL responses, and (iii) to stimulate the secretion of  $\gamma$  interferon by resting peripheral blood lymphocytes (M.K.G., unpublished results). It can also synergize with low concentrations of recombinant IL-2 in the latter two assays and in causing the proliferation of resting peripheral blood lymphocytes. In view of its production by peripheral blood lymphocytes and its diverse actions on lymphoid cells, it appears that CLMF constitutes a new interleukin. We propose that CLMF be

given the provisional designation IL-12. The availability of recombinant CLMF will now make possible a broader and more detailed characterization of its biology.

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# Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

**Purpose:** Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8<sup>+</sup> T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.

**Patients and Methods:** Patients were HLA-A2-positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.

**Results:** Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a

complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8<sup>+</sup> T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma-producing T cells against Melan-A ( $P = .015$ ) after vaccination, but not against an Epstein-Barr virus control peptide ( $P = .86$ ). There was a correlation between the magnitude of the increase in Melan-A-specific cells and clinical response ( $P = .046$ ).

**Conclusion:** This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.

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MOST MELANOMA tumors express antigens that can be recognized by CD8<sup>+</sup> T cells.<sup>1,2</sup> Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.<sup>3</sup> Specific CD8<sup>+</sup> T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.<sup>4</sup>

The optimal immunization strategy for inducing tumor antigen-specific CD8<sup>+</sup> effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived<sup>5,6</sup> and bone marrow-derived<sup>7</sup> dendritic cells (DCs) have been loaded with

melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN- $\gamma$ )-producing cytolytic effector cells.<sup>8-10</sup> Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors<sup>11,12</sup> and provision of exogenous IL-12, either alone<sup>13</sup> or combined with tumor antigen-based vaccines,<sup>14-19</sup> can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.<sup>20</sup> The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.<sup>21</sup> The effective range of doses indicated that a straight dose of 4  $\mu$ g might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1<sup>3</sup> peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2-positive patients with

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advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

## PATIENTS AND METHODS

### Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rIL-12.<sup>4</sup> The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2-positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status  $\geq 70$ , and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, *Candida*, and *Trichophyton*, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

### Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis, 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.<sup>22</sup> Only 45% were positive for at least one recall antigen (mumps, *Candida*, or *Trichophyton*) by DTH skin testing.

### RT-PCR Analysis

RNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.<sup>21</sup> Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide-stained agarose gel. No formal quantitation was performed.

### Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at  $40 \times 10^6$  cells/mL. At least  $10 \times 10^6$  cells from each sample were cryopreserved to prepare CD8<sup>+</sup> and CD8<sup>-</sup> fractions for subsequent correlative immunologic studies. The Melan-A<sub>27-35</sub> peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at

Table 1. Patient Characteristics

Patient Characteristic	Patients (n = 20)	
	No.	%
Age, years		
Median	58	
Range	35-79	
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2		None
$\geq 3$	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy		None
Adjuvant IFN- $\alpha$	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN- $\alpha$ , interferon alpha-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

\*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

-80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20  $\mu$ mol/L in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10  $\mu$ mol/L; target number of PBMCs,  $10^6$ ) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from  $78$  to  $100 \times 10^6$ .

rIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10  $\mu$ g under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rIL-12 was loaded into 3-mL syringes and used within 4 hours. rIL-12 (4  $\mu$ g) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rIL-12 for each cycle.

### Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

lesions; a partial response (PR) was defined as  $\geq 50\%$  reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

### CD8<sup>+</sup> T-Cell Preparation

CD8<sup>+</sup> and CD8<sup>-</sup> fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. CD8<sup>+</sup> T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound CD8<sup>-</sup> fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific CD8<sup>+</sup> T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, CD8<sup>+</sup> cells were thawed from each time point and pooled, pulsed with 50  $\mu\text{mol/L}$  Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with beta<sub>2</sub>-microglobulin, irradiated (3,000 rad), washed, and plated at  $2 \times 10^6$  cells/well in 24-well plates. CD8<sup>+</sup> T cells were thawed and cultured with the irradiated CD8<sup>-</sup> cells at  $4 \times 10^5$  cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A-pulsed irradiated CD8<sup>-</sup> cells. After an additional 5 days the cells were collected and tested.

### ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15  $\mu\text{g/mL}$  of antihuman IFN- $\gamma$  antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and CD8<sup>+</sup> T cells, either freshly thawed at  $5 \times 10^4$  cells/well or after in vitro expansion at  $5 \times 10^5$  cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing-deficient cell line, American Type Culture Collection no. CRI 1992) were pulsed for 1 hour at 37°C with 50  $\mu\text{mol/L}$  peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of CD8<sup>+</sup> T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5  $\mu\text{g/mL}$ ) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN- $\gamma$  antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin-alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN- $\gamma$  in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

### Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired *t* test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided *t* test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

## RESULTS

### Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

### Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

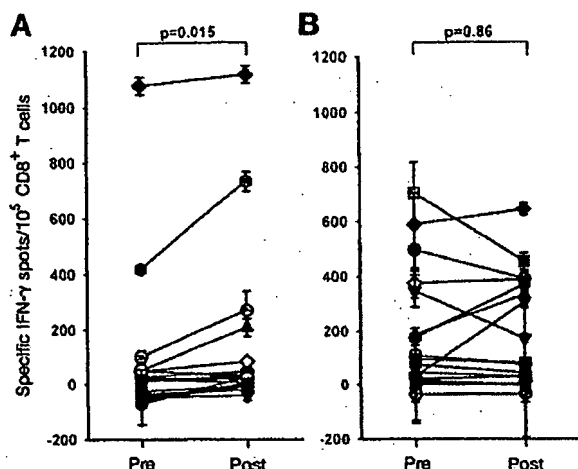


Fig 1. Interferon gamma ELISpot frequencies by CD8<sup>+</sup> T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired *t* test.

#### Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN- $\gamma$  ELISpot assay was used to monitor the immune response to immunization. Cryopreserved CD8<sup>+</sup> T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A-specific CD8<sup>+</sup> T cells before vaccination, with as high as 1% of CD8<sup>+</sup> cells responding to this peptide. These T cells were functional because they produced IFN- $\gamma$ . The majority of patients showed an increase in the frequency of Melan-A-specific cells after immunization ( $P = .015$ ). In contrast, the frequencies of specific CD8<sup>+</sup> T cells responding to the EBV peptide did not vary significantly overall ( $P = .86$ ). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A-specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A-specific T cells for the clinical responders was  $112 \pm 45$  and for nonresponders was  $26 \pm 16$ , indicating that a greater absolute increase in Melan-A-specific T cells was associated with tumor regression ( $P = .046$ ).

#### Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known

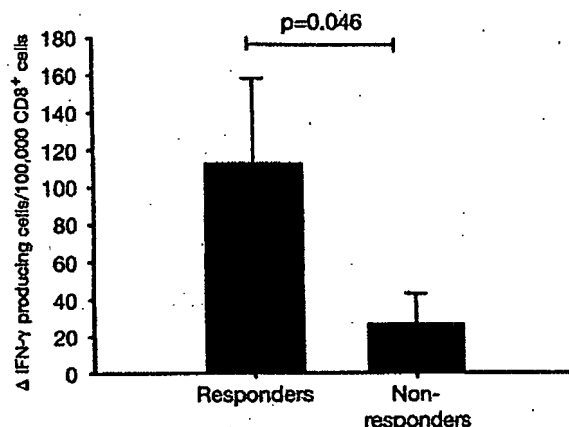


Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired *t* test.

negative prognostic factor,<sup>23</sup> survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH ( $P = .005$ ). In addition, the median survival for patients who experienced a significant increase in Melan-A-specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A-specific cells (Fig 3C;  $P = .120$ ).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A-specific CD8<sup>+</sup> T cells before immunization correlated with either outcome. The median pretreatment Melan-A-specific T cell frequency was 23 in clinical nonresponders and -26 in responders. To increase the sensitivity of the assay to detect Melan-A-specific T cells, an in vitro expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A-specific T cell frequencies after in vitro expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A-specific T cells and clinical outcome.

#### Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A-negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for

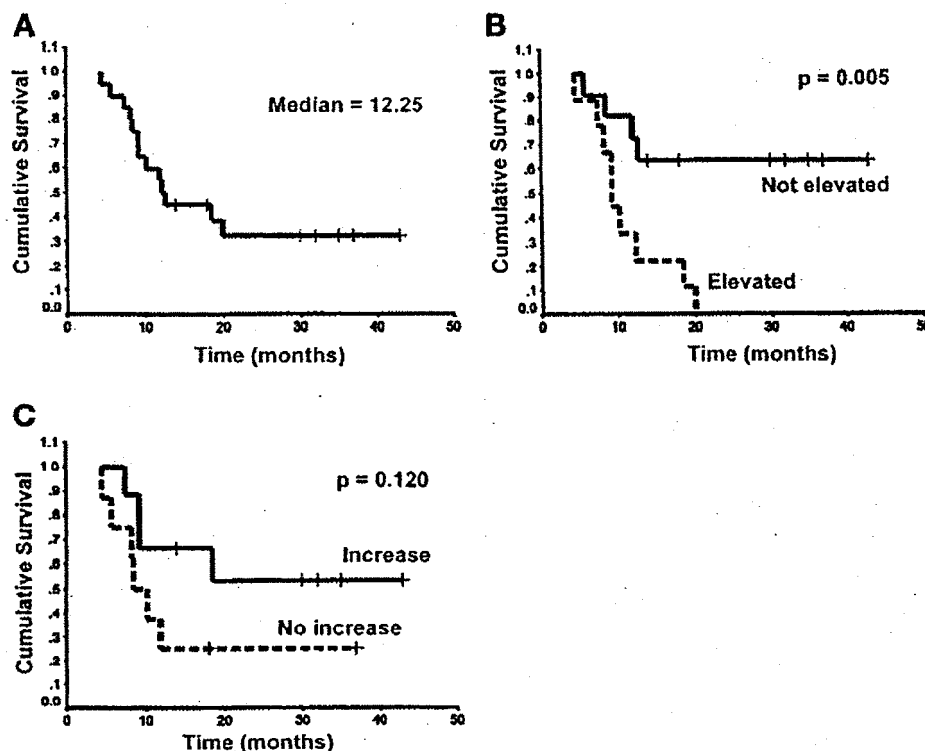


Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing CD8<sup>+</sup> T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

#### DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2-positive patients with advanced melanoma. We observed a significant increase in Melan-A-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended in vitro culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results

support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.<sup>24</sup>

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved CD8<sup>+</sup> T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A-reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified CD8<sup>+</sup> T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and natural killer T cells that could have produced IFN- $\gamma$  in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A-specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (P)	Correlation With Survival (P)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pre†	.290	.644
Strong Melan-A pre-Rx‡	.644	.481
Increased Melan-A post versus pre†	.046	.120
Strong in vitro expansion of Melan-A§	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two sided), except the differences between pre- and posttreatment, which were determined using an unpaired *t* test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

\*At least 90 spots per  $10^5$  CD8<sup>+</sup> T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired *t* test between clinical responders and nonresponders.

‡At least 40 spots per  $10^5$  CD8<sup>+</sup> T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per  $10^5$  CD8<sup>+</sup> T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous CD8<sup>+</sup> cells and IL-2.

PBMC + rhIL-12 and in other trials<sup>21,25</sup> in which in vitro expansion had been performed before assessment of IFN- $\gamma$  production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells in vivo.

High frequencies of Melan-A-specific, IFN- $\gamma$ -producing CD8<sup>+</sup> T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A-specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,<sup>26</sup> those cells had a naïve surface phenotype and did not produce high levels of IFN- $\gamma$ . What did correlate with clinical response in our current study is a meaningful increase in Melan-A-specific T cells posttreatment. These increases were modest (a net gain of 112 spots per  $10^5$  CD8<sup>+</sup> T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in

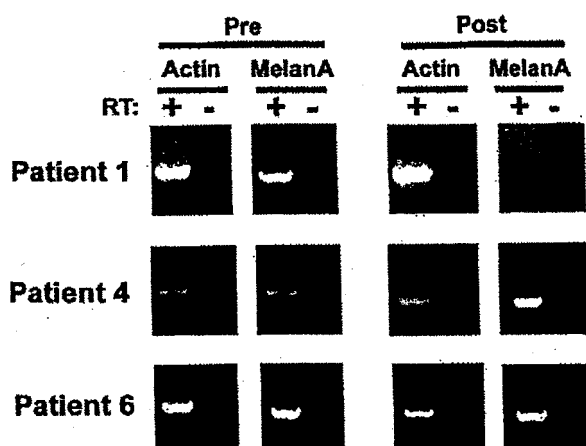


Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

T-cell frequencies using standard assays have been observed in other studies.<sup>27</sup>

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A-specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies<sup>28</sup> and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.<sup>29,30</sup> Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells in vivo.

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## Vaccination with Mage-3A1 Peptide-pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

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### Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin,  $3 \times 10^6$  DCs each subcutaneously and intradermally, followed by two intravenous injections of  $6 \times 10^6$  and  $12 \times 10^6$  DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1-specific CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and CD8<sup>+</sup> T cell infiltration, whereas nonregressing lesions lacked CD8<sup>+</sup> T cells as well as Mage-3 mRNA expression. This study proves the principle that DC "vaccines" can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active CD8<sup>+</sup> CTL-tumor cell interaction in situ as well as escape by lack of tumor antigen expression.

**Key words:** dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

**I**t is now established that the immune system has cells, particularly CD8<sup>+</sup> CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),<sup>1</sup> "nature's adjuvant" for eliciting T cell immunity (3). Another is that

tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimelanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

<sup>1</sup>Abbreviations used in this paper: CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.



antigens but lack full T cell-stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen-loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, *immature* DCs have been employed (9-11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of *mature* and *stable* DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide-pulsed mature DCs reliably enhance Mage-3A1-specific CD8<sup>+</sup> and recall CD4<sup>+</sup> T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1-specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC-mediated immunization and were accompanied by CD8<sup>+</sup> T cell infiltration, we propose that the induced Mage-3A1-specific CTLs are active *in vivo*.

## Materials and Methods

### Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/Union Internationale Contre Cancer pTNM staging system) that was not curable by resection and was progressive despite chemo(immuno)therapy. Further inclusion criteria were an expected survival  $\geq 4$  mo, Karnofsky index  $\geq 60\%$ , age  $\geq 18$  yr, measurable disease, HLA-A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosurea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was *not* required. Important exclusion criteria were active central nervous system (CNS) metastasis, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen/paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

### Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, NY) and performed under supervision of its Office of Clinical Trials Management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits 1, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

### Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF- $\alpha$  had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis ( $\geq 10^{10}$  nucleated cells) were isolated on Lymphoprep<sup>TM</sup> (Nycomed Pharma) and divided into three fractions. The first fraction of  $10^9$  PBMCs was cultured on bacteriological petri dishes (Cat. #1005; Falcon Labware) coated with human Ig (100  $\mu\text{g}/\text{ml}$ ; Sandoglobulin<sup>TM</sup>; Sandoz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 20  $\mu\text{g}/\text{ml}$  gentamicin (Refobacin 10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of  $3 \times 10^8$  PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test I. Adherent monocytes were cultured in 1,000 U/ml GM-CSF ( $10 \times 10^7$  U/mg; Leukomax<sup>TM</sup>; Novartis) and 800 U/ml IL-4 (purity  $>98\%$ ;  $4.1 \times 10^7$  U/mg in a bioassay using proliferation of human IL-4R<sup>+</sup> CTLL; CellGenix; expressed in *Escherichia coli* and produced under good laboratory practice conditions but verified for good manufacturing practice [GMP] safety and purity criteria by us) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP-rhu TNF- $\alpha$  (purity  $>99\%$ ;  $5 \times 10^7$  U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15) (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the

Multitest Merieux was >10 mm; both purchased from the Bacterial Vaccines Department of the Statens Serum Institute, Copenhagen, Denmark). The recall antigen was added at 10 µg/ml for the last 24 h, and the Mage-3A1 peptide was added at 10 µM directly to the cultures for the last 8 h (if immunity to recall antigens was strongly boosted, the dose of recall antigen was reduced to 1.0 or 0.1 µg/ml or was omitted for the intravenous DC injections to avoid a cytokine release syndrome). On day 7, mature DCs were harvested, resuspended in complete medium, washed, and pulsed once more with Mage-3A1 peptide (now at 30 µM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but no recall antigen); DCs that served as negative control in the DTH tests were not pulsed at all. An aliquot of the DCs to be used for vaccinations was analyzed as described (13) to assure that functionally active and mature DCs were generated. The features of the DCs are described in Results. Release criteria were typical morphology (>95% nonadherent veiled cells) and phenotype (>95% HLA-DR<sup>+</sup>CD86<sup>+</sup>CD40<sup>+</sup>CD25<sup>+</sup>CD14<sup>-</sup> and >65% homogeneously CD83<sup>+</sup>).

#### Immunization Schedule

A total of five vaccinations (three into the skin followed by two intravenously) with antigen-pulsed DCs were given at 14-d intervals (Table II). This design was chosen to explore the toxicity and efficacy of various routes in this trial. For vaccinations 1–3,  $3 \times 10^6$  DCs were given subcutaneously at two sites ( $1.5 \times 10^6$  DCs in 500 µl PBS per site) and  $3 \times 10^6$  intradermally at 10 sites ( $3 \times 10^5$  DCs in 100 µl PBS per site). The injection sites were the ventromedial regions of the upper arms and the thighs close to the regional lymph nodes and were rotated clockwise. Limbs where draining lymph nodes had been removed and/or irradiated were excluded. For intravenous vaccinations 4 and 5, a total of 6 and  $12 \times 10^6$  antigen-pulsed DCs (resuspended in 25 or 50 ml PBS plus 1% autologous plasma) was administered over 5 and 10 min, respectively. Premedication with an antipyretic (500 mg acetaminophen/paracetamol p.o.) and an antihistamine (2.68 mg clemastinhydrogenfumarat i.v.) was given 30 min before intravenous DC vaccination.

#### Evaluation of Immune Status

**Recall Antigen-specific Proliferation and Cytokine Production.** PBMCs were cultured in triplicate at two dose levels ( $3 \times 10^4$  and  $1 \times 10^5$  PBMCs/well) plus or minus TT or tuberculin (at 0.1, 1, and 10 µg/ml) and pulsed on day 5 with [<sup>3</sup>H]thymidine for 12 h. In all cases, the highest cpm were obtained with the highest doses of PBMCs and antigen and are shown in Fig. 2. IL-4 and IFN-γ levels were measured in culture media by ELISA (Endogen, Inc.). In a separate plate, staphylococcal enterotoxin (SEA; Serva) was added at 0.5, 1, and 5 ng/ml, and proliferation was assessed after 3 d to provide a positive control for helper T cell viability and responsiveness.

**Enzyme-linked Immunospot Assay for IFN-γ Release from Single Antigen-specific T Cells.** To quantitate antigen-specific, IFN-γ-releasing, Mage-3A1-specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (16). PBMCs ( $10^5$  and  $5 \times 10^5$ /well) or in some cases CD8<sup>+</sup> or CD4<sup>+</sup> T cells (isolated by MACS<sup>™</sup> according to the manufacturer, Miltenyi Biotec) were added in triplicate to nitrocellulose-bottomed 96-well plates (MAHA S4510; Millipore Corp.) precoated with the primary anti-IFN-γ mAb (1-D1K; Mabtech) in 50 µl ELISPOT

medium (RPMI 1640 and 5% heat-inactivated human serum) per well. For the detection of Mage-3A1-reactive T cells, the APCs were irradiated T2A1 cells (provided by P. van der Bruggen, Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed with MHC class I-restricted peptides (Mage-3A1 peptide and the HIV-1 p17-derived negative control peptide GSEELRSLY) added at  $7.5 \times 10^4$ /well (final volume 100 µl/well). After incubation for 20 h, wells were washed six times, incubated with biotinylated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, and stained with Vectastain Elite kit (Vector Labs.). For detection of TT-reactive T cells, TT was added at 10 µg/ml directly to the PBMCs ( $1$  or  $5 \times 10^5$  PBMCs/flat-bottomed 96-well plate). Assays were performed on fresh PBMCs. Spots were evaluated and counted using a special computer-assisted video imaging analysis system (Carl Zeiss Vision) as described (16).

**Semiquantitative Assessment of CTL Precursors.** The multiple microculture method developed by Romero et al. (17) was used to obtain a semiquantitative assessment of CTLp (precursors) specific for Mage-3A1 peptide. Aliquots of frozen PBMCs were thawed and assayed together. CD8<sup>+</sup> T cells were isolated with magnetic microbeads (MACS<sup>™</sup> separation columns; Miltenyi Biotec) and seeded at  $10^4$ /well in 96-well round-bottomed plates in RPMI 1640 with 10% heat-inactivated human serum. The CD8<sup>+</sup> PBMCs were pulsed with peptide Mage-3A1 or the influenza PB1 control peptide VSDGGPNLY (10 µg/ml; 30 min at room temperature), irradiated (30 Gy from a cesium source), and added as an APC population at  $10^5$ /well together with IL-2 (10 IU/ml final) and IL-7 (10 ng/ml final) in a total volume of 200 µl/well. On day 7, 100 µl fresh medium was substituted, and peptide Mage-3A1 or PB1 (1 µg/ml final) and IL-2 (10 U/ml) was added. On day 12, each microwell was divided into three equal samples to test cytolytic activity in a standard 4-h <sup>51</sup>Cr-release assay on peptide-pulsed (10 µg/ml for 1 h at 37°C) T2A1 cells, nonpulsed T2A1 cells, and K562 target cells, respectively. All of the assays were performed with an 80-fold excess of nonlabeled K562 to block NK activity. Microwells were scored positive if lysis of T2A1 targets with peptide minus lysis without peptide was ≥12% and this specific lysis was greater than or equal to twice the lysis of T2A1 targets without peptide plus six as described (18). We aimed at testing 30 microwells of  $10^4$  CD8<sup>+</sup> T cells. Therefore, 1/30 positive wells equals at least one CTLp in  $3 \times 10^5$  (i.e., 30 wells at  $10^4$  CTLp per well) CD8<sup>+</sup> T cells (corresponding to  $\sim 3 \times 10^6$  PBMCs).

**DTH.** DTH to Mage-3A1 peptide was assessed by intradermal injection at two sites of each  $3 \times 10^5$  Mage-3A1 peptide-loaded DC in 0.1 ml PBS. Negative controls were nonpulsed autologous DCs in 0.1 ml PBS and 0.1 ml PBS. DTH to seven common recall antigens (Multitest Merieux) including TT and tuberculin was performed on visits 1, 5, and 8 (Table II).

#### Assessment and Analysis of Tumor Tissue

For recruitment into the study, Mage-3 gene expression in at least one metastatic deposit had to be demonstrated by RT-PCR as described (14). Accessible superficial skin metastases were removed whenever possible after the vaccinations and subjected to Mage-3 RT-PCR as well as routine histology and immunohistochemistry (to characterize cellular infiltrates).

#### Statistical Analysis

For analysis of the immune response, pre- and postimmunization values were compared by paired *t* test after logarithmic transformation of the data. Significance was set at *P* < 0.05.

## Results

### Patient Characteristics

All 13 patients were HLA-A1<sup>+</sup>, had proven Mage-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemimmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

### Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in

appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR<sup>+++</sup>CD86<sup>+++</sup>CD40<sup>+</sup>CD25<sup>+</sup>CD14<sup>-</sup>), and acted as strong stimulators of an MLR at DC/T cell ratios of  $\leq 1:300$  (13). Most (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with Mage-3A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

### Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. Patients' Characteristics, Status before DC Vaccination, and Response to DC Vaccination

Patient code	Sex-Age	Onset stage IV	Previous therapy	Metastases at study entry <sup>a</sup>							Clinical Response	Survival
				regional		distant					14 days after the 5 <sup>th</sup> vaccination	
				skin	LN	Skin	LN	Lung	Liver	Other		
Patients with objective tumor regression												
04	M48	1/98	PCI				1/15	m/30		CNS 2/12	complete regression of all but 1 lung metastasis, overall progression	10 + >9
06	F61	10/97	CI			3/19		m/20	2/15		complete regression <sup>b</sup> of 1 lung + 4 s.c. <sup>a</sup> metastases, overall progression	6 + >16
07	F48	6/97	C			1/7				ovary 1/30 bone 3/70	complete regression <sup>b</sup> of 1 lung <sup>a</sup> + 2 s.c. <sup>a</sup> metastases, overall progression	13 + 12†
08	M67	11/97	PC		2/54		m/30	2/25	2/20		complete regression <sup>b</sup> of lung + liver + 4 s.c. <sup>a</sup> metastases, overall progression <sup>c</sup>	8 + 3†
09	F43	5/98	C					1/25		metast. 1/45 bone 2/15	Partial regression of 1 lung metastasis, overall progression	4 + >11
12	M54	9/98	CI			2/30	m/15	m/20			partial regression of axillary LN metastases, overall progression	28 + >9
Patients without objective tumor regression												
02	F73	5/98	PCI	50/40			m/28	m/15	m/35	pancr. 1/10	continuous progression	18 + 5†
05	F49	10/97	CI		1/10		2/15	m/18			continuous progression	5 + >17
10	M62	8/98	C	6/75		1/10					continuous progression	1 + 6†
11	F72	7/98	C		m/25	2/15		3/12		bone 1/14	continuous progression	4 + 9†
13	M34	12/97	CI				1/25		8/25	bone 1/14	continuous progression	12 + 5†

Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen.

Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed.

Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases).

Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. †Patient deceased.

<sup>a</sup>CNS metastases were regressing at study entry after gamma knife treatment.

<sup>b</sup>Developed (in part) after study entry.

<sup>c</sup>Determined by autopsy.

<sup>d</sup>Sudden death from bleeding into CNS metastasis on visit 8.

<sup>e</sup>The regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8.

medast., mediastinum; pancr., pancreas.

Table II. Study Design

Activities	Screen	Leuka pheresis	Vacc. #1 3 Mio s.c. 3 Mio i.d.	Vacc. #2 3 Mio s.c. 3 Mio i.d.	Vacc. #3 3 Mio s.c. 3 Mio i.d.	Vacc. #4 6 Mio i.v.	Vacc. #5 12 Mio i.v.	Final Evaluation
Clinical visit	1	2	3	4	5	6	7	8
Day	-28/-14	-9	+1	+14	+28	+42	+56	+70
Vaccination			X	X	X	X	X	
Multitest Merieux	X				X			X
DTH to Mage-3A1 peptide-loaded DC			X		X		X	
Recall-antigen proliferation		X						X
CTLp analysis		X				X		X
ELISPOT Mage-3A1		X	X	X	X	X	X	X
ELISPOT recall antigen		x	x	x	x	x	x	x

X, prespecified in the protocol as obligatory; x, optional.

neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration >10 mm in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to 40°C) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63% and by sonography in 83% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

#### Immunological Responses

**Boosting of Recall Antigen-specific Immunity.** PBMCs that had been frozen before vaccination and 14 d after vaccination 5 were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) ( $P < 0.004$ ; Fig. 2). Supernatants from the proliferative assays contained large amounts of IFN- $\gamma$  (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN- $\gamma$ /IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN- $\gamma$  ELISPOT analysis. We found an increase after the intracutaneous vaccinations ( $P < 0.02$ ) but a peculiar decrease after the intravenous vaccinations ( $P < 0.008$ ; Fig. 3). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.

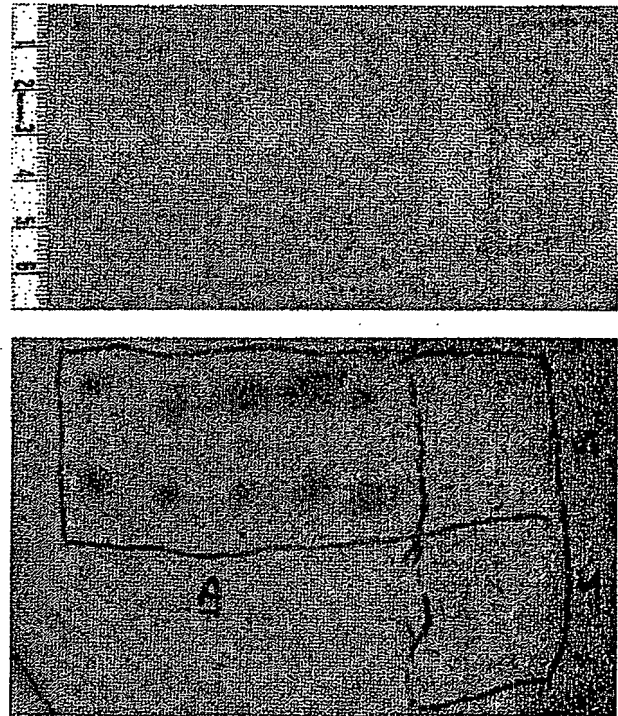


Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the 10 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration >10 mm in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).

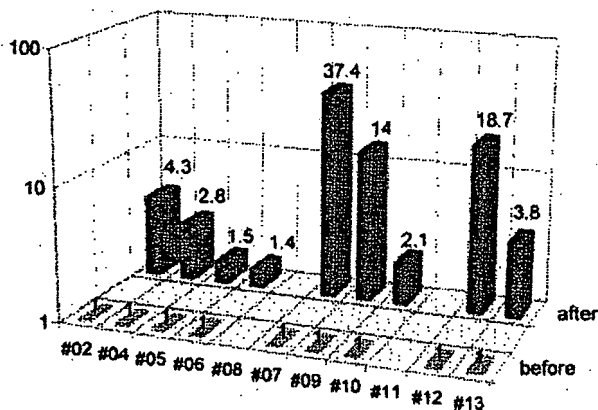


Figure 2. Recall antigen-specific immunity (tuberculin in patient 10; TT in all others) as assayed by antigen-specific proliferation. The cpm values determined after therapy (14 d after vaccination 5) are shown as multiples of pretherapy cpm values. Absolute cpm (cpm with recall antigen minus cpm without antigen) after therapy was 68,917 in patient 02, 85,225 in patient 04, 16,759 in patient 05, 7,913 in patient 06, 16,367 in patient 07, 107,923 in patient 09, 22,790 in patient 10, 4,507 in patient 12, and 1,831 in patient 13 (SEM for all measurements was <20%). Patients 08 and 11 could not be evaluated due to shortage of cells after therapy.

**Expansion of Mage-3A1-specific CTLp.** Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1-specific CTLp as indicated by the increase (mean, eightfold;  $P < 0.008$ ) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ( $P < 0.0013$ ) but then decreased after the two additional intravenous vaccinations in all but one of these patients ( $P < 0.026$ ). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 Influenza peptide that served as a specificity control (not shown).

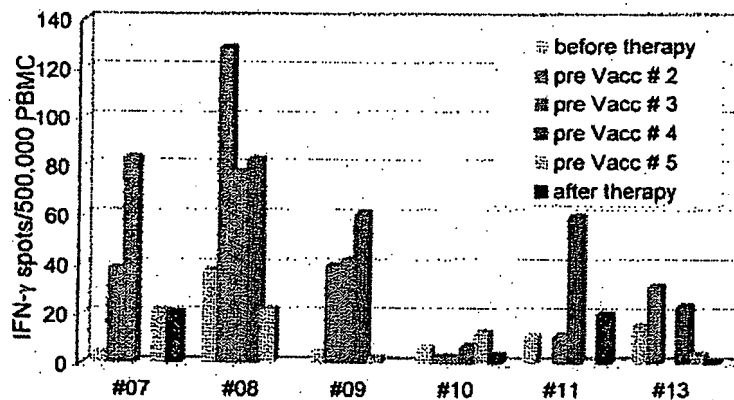


Figure 3. Kinetic analysis of immunity to recall antigens as assessed by TT-specific IFN- $\gamma$  ELISPOT (SEM for all measurements was <20%). Blood was drawn (see Table II, Study Design) before the first DC vaccination and then every 14 d just before administration of the next DC vaccination (e.g., pre Vacc # 2 means immediately before vaccination 2, i.e., 14 d after vaccination 1), and finally after therapy. Time points at which vaccinations were not performed lack bars. Note the increase after the intracutaneous vaccinations and the decline upon the two vaccinations after intravenous ones. Patient 10, who received tuberculin-pulsed DCs, exhibited no significant change in the TT-specific IFN- $\gamma$  ELISPOT as expected.

**ELISPOT Analysis for IFN- $\gamma$ -releasing, Mage-3A1-specific T Cells.** We also tried to detect Mage-3A1-specific CTL effectors in uncultured fresh, nonfrozen PBMCs by performing ELISPOT analyses at 14-d intervals on all patients. A significant increase of Mage-3A1-reactive IFN- $\gamma$  spot-forming cells was apparent only in patients 07 and 09 after the first and second vaccinations, respectively, but the frequency of Mage-3A1-specific effectors was very high (~5,000 and 10,500/10<sup>7</sup> CD8<sup>+</sup> T cells; not shown).

**DTH Test to Mage-3A1 Peptide-loaded DCs.** Tests of DTH to Mage-3A1 peptide-loaded DCs yielded erythema and/or induration (>5 mm diameter) in 7/11 patients (not shown). The results were, however, equivocal due to the frequently observed background to nonpulsed DCs (up to 10 mm in diameter) and the variability from test site to test site.

### Clinical Responses

At the end of the trial, i.e., ~2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07), it was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of CD8<sup>+</sup> T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 mRNA<sup>+</sup>. However, all of the samples removed at the end (two and six, respectively) were Mage-3 mRNA<sup>-</sup>, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of CD8<sup>+</sup> T cells was not found in any of these lesions.

### Discussion

In deciding on the source of DCs for this phase I trial, we selected mature, monocyte-derived DCs for the follow-

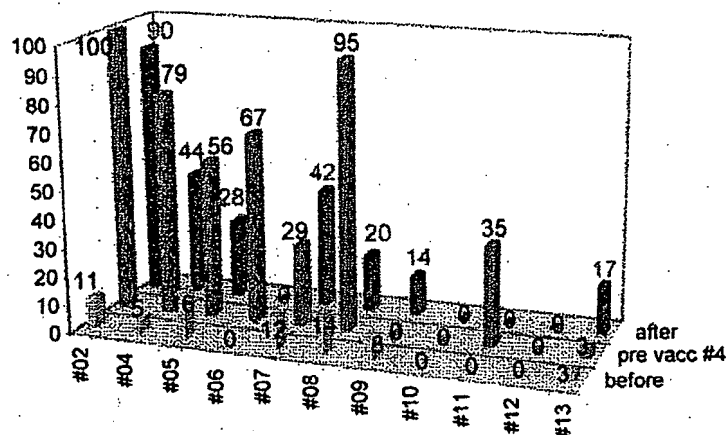


Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axis and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

ing reasons. Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses in vitro (reference 22 and Jönuleit, H., A. Gleseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in

lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1-specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per  $10^7$  CD8<sup>+</sup> T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 CD8<sup>+</sup> T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1-specific T cells are CD8<sup>+</sup>

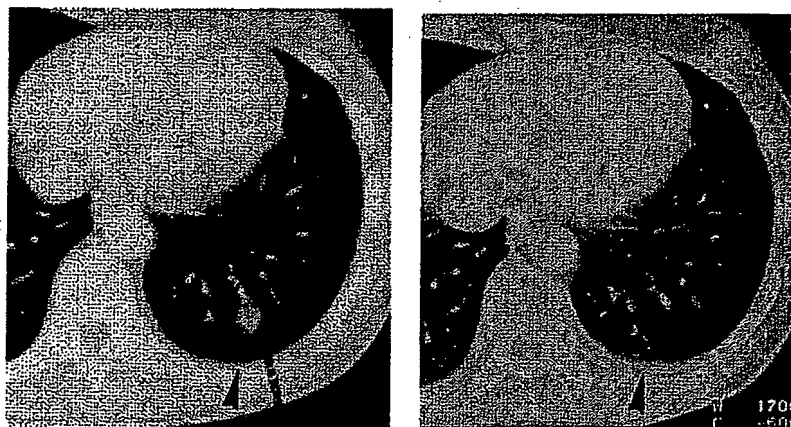
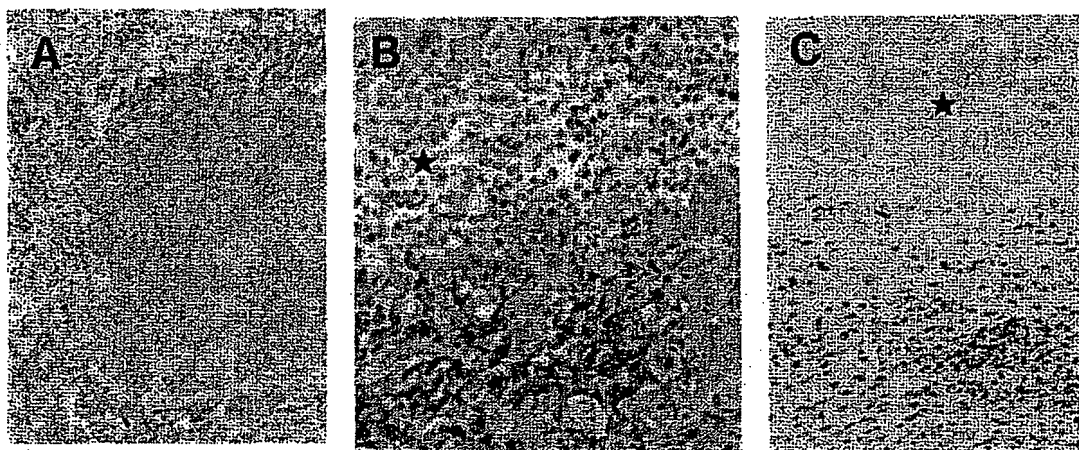


Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.





**Figure 6.** Regressing subcutaneous metastases in patient 06 display a CD8<sup>+</sup> lymphocytic infiltrate (alkaline phosphatase/antialkaline phosphatase immunohistochemical staining with anti-CD8 mAb) that surrounds (A) and infiltrates (B) the tumor. Areas of damaged (B, ★) and necrotic (C, ★) melanoma cells are obvious in the vicinity of the CD8<sup>+</sup> T cell infiltrate. The metastasis expressed Mage-3, as demonstrated by RT-PCR (data not shown). Magnifications: A, 100; B, 250; C, 160.

T cells and DTH assays typically detect primed CD4<sup>+</sup> T cells, we suspect that DTH to MHC class I peptide-pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific CD8<sup>+</sup> T cell-mediated immunity.

In contrast, we found sizable expansions of Mage-3A1-specific CTL precursors in PBMCs from a majority (8/11) of patients ( $P < 0.008$ ; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1-specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies ( $>5,000/10^7$  CD8<sup>+</sup> T cells). Perhaps active CD8<sup>+</sup> effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations ( $P < 0.0013$ ) and then decreased ( $P < 0.026$ ) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3-reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were all progressive despite standard chemotherapy and even chemimmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1-specific CTLs. This interpretation is supported by the heavy infiltration with CD8<sup>+</sup> T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 mRNA<sup>+</sup> (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen-negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1-specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(immuno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between

CD8<sup>+</sup> CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing

variables such as DC maturational state, route, dose, and schedule or by improving the short life span of DCs *in vivo* (31, 32); at the level of the T cell, e.g., by providing melanoma-specific CD4<sup>+</sup> T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant.

Docket No.:

Serial No .

Group Art Unit:

Filing Date:

Examiner:

For:

DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I am an inventor of the above-identified patent application.
2. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.
3. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.
4. My scientific Curriculum Vitae is attached to and forms part of this Declaration.
5. I am familiar with the skin vascular permeability assay (Assay #64), which has been used by me and others under my supervision, to test the activities of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.
6. The skin vascular permeability assay, is also known in the art as the Miles assay and was first described by Miles and Miles in 1952 when they studied vascular responses to histamine (Miles A.A., and Miles E.M., J. Physiol., 1952, 118: 228-257, see Exhibit A). Since then it has been reliably used for identifying proinflammatory molecules.
7. Proinflammatory molecules can directly or indirectly cause vascular permeability by causing immune cells to exit from the blood stream and move to the site of injury or infection. These proinflammatory molecules recruit cells like leukocytes which includes monocytes, macrophages,

basophils, and eosinophils. These cells secrete a range of cytokines which further recruit and activate other inflammatory cells to the site of injury or infection. How leukocytes exit the vasculature and move to their appropriate destination of injury or infection is critical and is tightly regulated. Leukocytes move from the blood vessel to injured or inflamed tissues by rolling along the endothelial cells of the blood vessel wall and then extravasate through the vessel wall and into the tissues (see Exhibit B). This diapedesis and extravasation step involves cell activation and a stable leukocyte-endothelial cell interaction.

8. Hence, proinflammatory molecules are useful in treating infections, as local administration of the proinflammatory polypeptide would stimulate immune cells already present at the site of infection and induce more immune cells to migrate to the site, thus removing the infection at a faster rate. Examples of proinflammatory molecules that induce neutrophils to extravasate are MIP-1 and MIP-2. Other proinflammatory may be able to activate immune cells, as shown with the CXC chemokines activation of neutrophils, and the non-CXC chemokines which are chemotactic for T lymphocytes (Baggiolini *et al.*, Adv Immunology 1994; 55:97-179 see Exhibit C).

Inappropriate expression of proinflammatory molecules may cause an abnormal immune cell response and lead tissue destruction. Examples of such abnormal immune responses include at least the following conditions: psoriasis, inflammatory bowel disease, renal disease, arthritis, immune-mediated alopecia, stroke, encephalitis, Multiple Sclerosis, hepatitis, and others. Therefore, inhibitors of such proinflammatory molecules find use in the treatment of these conditions. Further, proinflammatory molecules with angiostatic properties are useful in the inhibition of angiogenesis during abnormal wound healing or abnormal inflammation during infection. Further, proinflammatory molecules that are also angiostatic are useful in treating tumors, by inhibiting the neovascularization that accompanies tumor growth (Strieter RM. *et al.*, J. Biol. Chem. 1995; 270: 27348-27357 see Exhibit D). Administration of the proinflammatory polypeptide, either alone or in combination with another angiostatic factor such as anti-VEGF, would be useful for limiting or reducing tumor growth.

9. The Skin Vascular Permeability Assay was used to identify such proinflammatory and immune related molecules. Miles and Miles described this assay initially in 1952, in their work on vascular response to histamine (Miles A.A., and Miles E.M., J. Physiol. 1952; (118) 228-257 *supra*). Miles and Miles solved the critical variables in the assay, such as performing the intradermal injection, where to inject, effects of temperature, effects of dosage, and effects of anesthetic used. Using this assay, Miles and Miles proved that histamine increased the capillary permeability in the skin, thus allowing cells to exit the vasculature. The assay was used qualitatively until other investigators quantitated it by

extracting the amount of accumulated marker dye and measuring its absorbance at 620 nm (Udaka et al., Proc Soc Exp Biol Med 1970; (133) 1384-1387 see **Exhibit E**).

10. The Skin Vascular permeability assay was used in the clinic in determining if blood coagulation factor XIII (FXIII) could be used in treating Shonlein Henoch Purpura (SHP) (Hirahara K., et al., Thrombosis Res 1993; 71(2) 139-148 see **Exhibit F**). SHP is an immunovascular disease, characterized by hemorrhagic skin lesions, gastro-intestinal bleeding and hematuria. The physiology of SHP was undetermined, but destruction of FXIII by leukocytes that migrated into the area and secreted destructive proteases was believed to play a role. This hypothesis was tested by using antibodies raised to guinea pig endothelial cells. These antibodies were specific for endothelial cells and not for fibroblasts, and when injected into the skin of a guinea pig, caused increased vascular permeability. When FXIII was mixed with this antibody and injected, the marker dye showed less spreading, indicating that FXIII was suppressing the vascular permeability and did so in a dose dependant manner. The conclusion was that FXIII was stabilizing the microvasculature, leading to less permeability, and therefore FXIII may be useful in the treatment of SHP.

11. The Skin Vascular Permeability assay has been confirmed by alternate experimental methods. Senger et al., used the Skin Vascular Permeability assay as described by Miles and determined that a secreted factor they called VPF (later determined to be VEGF), caused vascular permeability (Senger D.R., et al., Science 1983; (219) 983-985 see **Exhibit G**). In these experiments VPF was subjected to the Skin Vascular Permeability assay, the sites of injection were analyzed by light and electron microscopy and VPF caused vascular permeability without damaging endothelial cells or causing mast cell degranulation.

12. Yeo et al., confirmed the viability of the Skin Vascular Permeability assay by correlating it with disassociation enhanced lanthanide fluoroimmunoassay (DELFI A) results (Yeo K.T., Clin. Chem 1992; (38) 71-75 see **Exhibit H**). VPF (VEGF) was first measured using the Skin Vascular Permeability assay by quantifying the amount of accumulated dye in the skin as described in Udaka et al., (supra). Anti-VPF antibodies were used to quantitate the amount of VPF in the DELFI A. This assay has increased sensitivity as the result is read by a fluorometer, instead of dye absorbance. The investigators found that the sensitivity of the DELFI A was greater than the Skin Vascular Permeability assay, but there was an excellent linear correlation ( $r^2 = 0.94$ ) between the two assays.

13. The Applicant's Skin Vascular Permeability assay was conducted using anesthetized Hairless guinea pigs. A sample of a purified PRO polypeptide or a conditioned media test sample was injected intradermally onto the backs of the test animals with 100  $\mu$ L per injection site. It was possible to have up to about 24 injection sites per animal. One mL of Evans Blue dye (1% in physiologic buffered saline), was injected intracardially as the marker dye. Blemishes at the injection sites were then measured (mm diameter) after 1 hr, 6 hrs and 24 hrs post injection. Animals were sacrificed at the appropriate time after injection, and each skin injection site was biopsied and fixed in paraformaldehyde. The biopsies were then prepared for histopathologic evaluation. Each site was evaluated for inflammatory cell infiltration into the skin. Sites with visible inflammatory cell inflammation were scored as positive. Polypeptides tested stimulated an immune response and induced mononuclear cell, eosinophil and PMN infiltration at the site of injection of the animal. Perivascular infiltrate at the injection site was scored as positive; no infiltrate at the site of injection is scored as negative.

An example of a positive reaction is shown in Exhibit I. The top row is injected with Interleukin-8 (IL-8) control and shows no increased vascular permeability. The 2<sup>nd</sup> row from the top is VEGF as a positive control. The 2 bottom rows show a positive result from a PRO polypeptide, performed in duplicate.

14. It is my opinion that the PRO polypeptide that shows activity in the Skin Vascular permeability assay has specific, substantial and credible utilities. In the experiments performed in the instant application, the results of the skin vascular permeability assay were further analyzed by histopathological examination to rule out inflammation due to endothelial cell damage or mast cell degranulation. Hence, the vascular permeability observed was not due to histamine release or endothelial cell damage. Examples of utilities include, enhancing immune cell recruitment to sites of injury or infection, or inhibitors to treat autoimmune diseases such as psoriasis etc. as discussed above. Furthermore, the angiogenic or angiostatic properties of proinflammatory molecules would also find practical utility in controlling tumorigenesis.

15. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further, that these statements are made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent granted thereon.

Date:

8/27/04

By:

Sherman Fong  
Sherman Fong, Ph.D.

## **Sherman Fong, Ph.D.**

Senior Scientist  
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### Education:

1978 - 1980 Postdoctoral Fellow in Immunology,      Research Institute of Scripps Clinic,  
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology,      University of California at  
San Francisco, San Francisco, California

1970 - 1975 Ph.D. in Microbiology,      University of California at  
Davis, California

1966 - 1970 B.A. in Biology/Microbiology,      San Francisco State  
University, San Francisco, California

### Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc.  
South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological  
Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco,  
California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical  
Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research,  
Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical  
Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical  
Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology  
and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Research Grant, NIH (1985)

Recipient: P.I., Research Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Committee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmacological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the **Biochemical Subteam for Protropin® Project team.**

Served as Immunologist on the **Met-less hGH and Dnase project teams**, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in 1989-1990 on the **CD4-IgG project team** carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte  $\beta 2$  integrins. Provided preclinical scientific data to **Anti-CD18 project team**



supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule  $\alpha 4\beta 1$  integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped established and responsible for maintaining current research collaboration with Roche-Nutley. Project transferred to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against  $\alpha 4\beta 7$  integrin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn's disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for  $\alpha 4\beta 7$ /MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc** reviewer on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as Scientific advisor with staff of the **Business Development Office** on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The  $\beta 2$  Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process -- Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process -- Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin  $\alpha 4 \beta 7$  Attenuates Inflammatory Bowel Diseases", in Symposium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.

U.S. Patent Number 5,068,177: Anti-idiotypic Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.

U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.

U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colitis, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen

U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood

U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood. U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura

US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood  
US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

Publications:

1. Scibienski R, Fong S, Benjamini E: Cross tolerance between serologically non-cross reacting forms of egg white lysozyme. *J Exp Med* 136:1308-1312, 1972.
2. Scibienski R, Harris M, Fong S, Benjamini E: Active and inactive states of immunological unresponsiveness. *J Immunol* 113:45-50, 1974.
3. Fong S: Studies on the relationship between the immune response and tumor growth. Ph D Thesis, 1975.
4. Benjamini E, Theilen G, Torten M, Fong S, Crow S, Henness AM: Tumor vaccines for immunotherapy of canine lymphosarcoma. *Ann NY Acad Sci* 277:305, 1976.
5. Benjamini E, Fong S, Erickson C, Leung CY, Rennick D, Scibienski RJ: Immunity to lymphoid tumors induced in syngeneic mice by immunization with mitomycin C treated cells. *J Immunol* 118:685-693, 1977.
6. Goodman JW, Fong S, Lewis GK, Kamin R, Nitecki DE, Der Balian G: T lymphocyte activation by immunogenic determinants. *Adv Exp Biol Med* 98:143, 1978.
7. Goodman JW, Fong S, Lewis GK, Kamin R, Nitecki DE, Der Balian G: Antigen structure and lymphocyte activation. *Immunol Rev* 39:36, 1978.
8. Fong S, Nitecki DE, Cook RM, Goodman JW: Spatial requirements of haptenic and carrier determinants for T-dependent antibody responses. *J Exp Med* 148:817, 1978.
9. Fong S, Chen PP, Nitecki DE, Goodman JW: Macrophage-T cell interaction mediated by immunogenic and nonimmunogenic forms of a monofunctional antigen. *Mol Cell Biochem* 25:131, 1979.
10. Tsoukas CD, Carson DA, Fong S, Pasquali J-L, Vaughan JH: Cellular requirements for pokeweed mitogen induced autoantibody production in rheumatoid arthritis. *J Immunol* 125:1125-1129, 1980.
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# VASCULAR REACTIONS TO HISTAMINE, HISTAMINE-LIBERATOR AND LEUKOTAXINE IN THE SKIN OF GUINEA-PIGS

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A substantial increase in capillary permeability is a feature of acute inflammation in bacterial infections. The present investigation is part of an attempt to prove an old hypothesis, namely, that this increase in permeability is mediated by histamine. A comparative study was made of histamine, of the histamine-liberator 48/80, a condensation product of *p*-methoxyphenylethylmethylamine and formaldehyde (Baltzly, Buck, de Beer & Webb, 1949), and of leukotaxine (Menkin, 1936, 1938*a, b*).

In animals with a recently injected vital dye in their blood, the intradermal injection of substances that increase permeability of the blood vessels is followed by an accumulation of dye at the site of injection, presumably due to the passage of an excess of dye-stained plasma into the tissue spaces. When the blood flow and the vascular bed of the skin are relatively constant, differences in the size and intensity of stained areas of skin reflect differences in vascular permeability, and may be used to investigate the properties of substances that increase permeability in this way. Our work was confined to the skin of guinea-pigs, partly because much is already known about skin reactions to toxins and other inflammatory agents, and partly because it is a tissue readily studied in the intact and unanaesthetized animal—an important consideration with phenomena which, like the passage of dye through vascular endothelium, are peculiarly dependent on the state of the blood vessels in the tissue under test.

## MATERIALS AND METHODS

Albino guinea-pigs, 300-450 g in weight, were used throughout. The skin of the trunk was depilated, after clipping away the hair, by a paste consisting of wheat flour, 350 g; talcum powder, 350 g; barium sulphide, 250 g; Castile soap powder, 50 g; and water. The depilated area was thoroughly washed with warm water.

*Detection of increased permeability.* Neither the intradermal injection nor the pricking-in of histamine or 48/80 produce in the depilated skin any measurable reaction indicating change in

vascular permeability of the skin to histamine. It is remarkably constant and is not used, given intravenously, referred to below as 48/80. It is of too-long application to the skin, becoming depilation, with nice results. Owing to the sensitivity of the skin, it is pinched into a fold by the underlying tissue.

In the absence of formaldehyde, the skin is mally stained in 10 h. of a substance that is not used. The best contrasts were obtained by intradermal injections were given in the knee joint in the sitting position, ventral mid-line; all the skin was blueing. The volume of the skin was measured with short diameter develops at the site of injection.

Skin reactions in the guinea-pig to injections with a no. 28 needle into animals under light anaesthesia. The dermal or made direct the numerous anastomoses illuminated and observed.

*Materials.* The acid-base. The specimen of the dimer, trimer. The leukotaxine was prepared by methods of Cullumbi described by Spector and amino-acid residues.

The behaviour of intradermal injection in the abdominal and thoracic skin is described as three layers and dermis which is as a dense white extensive plexus of hair follicles, and ink is injected in the tissue about 1-0 mm layer; at its base

HISTAMINE-  
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skin of the trunk was , 350 g; talcum powder, The depilated area was

nor the pricking-in of n indicating change in

vascular permeability; and there is no trace of the wheal that characterizes the reaction of human skin to histamine. In the skin of animals with circulating dye, however, both substances induce remarkably constant effects. A 5% solution of pontamine sky blue 6X ('pontamine blue') was used, given intravenously in the leg in doses of 65-75 mg/kg body weight. Animals so injected are referred to below as 'blued'. A few minutes after the injection of the dye the sites of wounds, of too-long application of depilating paste, and of recent careless or even firm manipulation of the skin, become blue. Though traumatic blueing of this kind commonly results from depilation, with nice judgement it is possible to depilate cleanly without damage to the skin. Owing to the sensitivity to trauma of blued animals, injections cannot be made into the skin pinched into a fold between thumb and finger; the skin must be steadied by gentle stretching over the underlying tissue.

In the absence of further interference, the skin of a blued animal becomes generally and maximally stained in 10 hr or more; but up to 6 hr after the intravenous dye, the intradermal injection of a substance that increases permeability results in a local increase in the intensity of blueing. The best contrasts were obtained within 1 hr of giving the dye. Unless otherwise stated, all our injections were given into the skin of the trunk posterior to the shoulder blade and anterior to the knee joint in the sitting animal, and omitting the thin skin about 30-40 mm on each side of the ventral mid-line; all solutions for injection were made up in 0.85% saline, which by itself induces no blueing. The volume injected was usually 0.1 ml., which initially raises a bleb 9-11 mm in diameter. With short-bevel no. 26 gauge needles, a small area of traumatic blueing 1-3 mm in diameter develops at the centre of the bleb.

Skin reactions in the ears of blued animals were induced either by free-hand intradermal injections with a no. 28 needle, or by injections with a mechanically manipulated glass micro-needle into animals under light bromethol (avertin) anaesthesia. The micro-injections were either intradermal or made directly into the lymphatic plexus of the ear, which is readily entered via one of the numerous anastomosing lymphatic channels at the margin of the ear. The ears were transilluminated and observed under  $\times 20$  and  $\times 40$  magnification.

**Materials.** The acid phosphate of histamine was used; amounts are cited as the weight of the base. The specimen of 48/80 (Wellcome Research Laboratories, U.S.A.) was probably in the form of the dimer, trimer and tetramer (Paton, 1951), with an average molecular weight of about 540. The leukotaxine was a single batch prepared by Dr J. H. Humphrey, by a combination of the methods of Cullumbine & Rydon (1946) and Spector (1951), and corresponded to the fractions described by Spector as active in inducing capillary permeability, containing eight to fourteen amino-acid residues. On this basis its molecular weight is of the order of 1500.

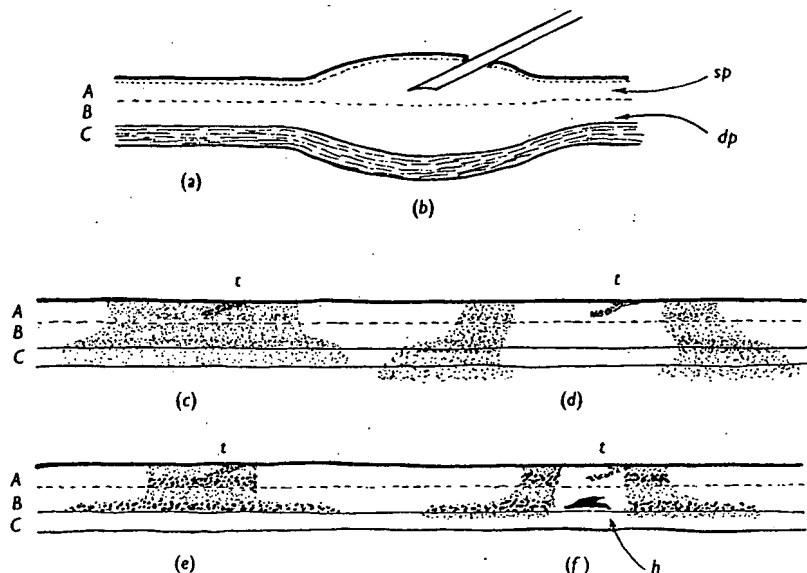
## RESULTS

*The mechanics of intradermal injection*

The behaviour of injected drugs is determined in part by the mechanics of intradermal injection. The skin of the trunk moves loosely over the underlying abdominal and thoracic muscles. For our purpose this movable skin may be described as three layers of about equal thickness (Text-fig. 1a). (A) Epidermis and dermis which together are about 1.0 mm thick, appearing on cross-section as a dense whitish layer, whiter in the deeper part; the dermis contains an extensive plexus of blood vessels (*sp*) round the main bodies of the glands and hair follicles, and a fine plexus of lymphatic channels, detectable when indian ink is injected into this region by a micro-needle. (B) A looser connective tissue about 1.0 mm thick, appearing on cross-section as a grey gelatinous layer; at its base, immediately above C, the panniculus carnosus, is a plexus

of blood vessels (*dp*) and a coarse scanty plexus of lymphatic channels, each joined to the corresponding upper plexus in *A* by relatively few vessels. (*C*) The panniculus carnosus, a muscle layer about 1.0 mm thick.

In making an intradermal injection, the depth of the needle-tip to some extent determines the depth at which the bulk of the injected fluid will spread, but not as completely as is generally supposed. The fluid spreads outwards, upwards and downwards to form a lenticular mass of wet tissue (Text-fig. 1*b*). When the needle tip is as low as the deepest part of *B*, the swelling of the skin



Text-fig. 1. Schematic sections of guinea-pig skin. (*a*) normal; (*b*) intradermal injection bleb; (*c*) blueing with low dose of histamine, showing traumatic blueing *t*, due to needle; (*d*) blueing with high dose of histamine showing central inhibition; (*e*) blueing with low dose of 48/80; (*f*) blueing with high dose of 48/80, showing central inhibition, and haemorrhage, *h*. *sp*, *dp* = superficial and deep plexus of blood vessels. For definition of layers *A*, *B* and *C*, see p. 229.

is due mainly to the distension of that layer, but when it lies in the middle of *B*, or in the dermis, both *B* and the dermis are equally permeated by the injection fluid. The initial diameter of the bleb is a simple function of volume injected; being linearly related to log. volume (Text-fig. 3*a-d*).

The initially domed injection-bleb of saline is scarcely visible after 3-4 hr. Some of the fluid is doubtless taken into the blood stream and some into the lymphatic channels; though, judging by the results of intradermal injection of dyes and indian ink, only a very small proportion of the injected substances escapes by the lymphatic channels during the first 2 hr. Other forces must be at work to account for the gradual disappearance of the bleb, which both decreases in thickness and spreads outwards. The diameter of a 0.1 ml. bleb,

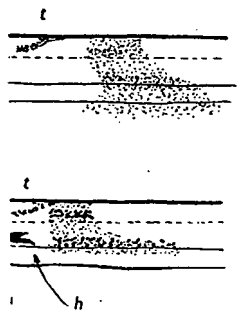
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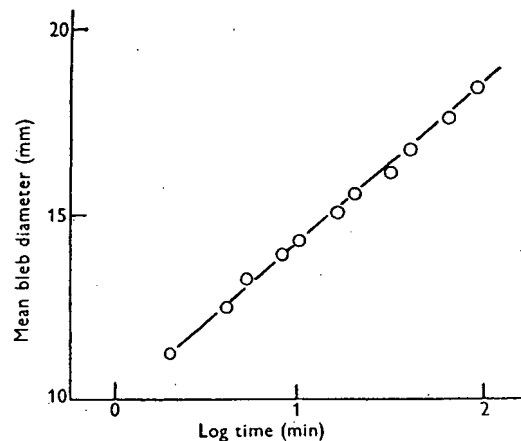
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measured under illumination by a very oblique beam of light, grows linearly with respect to log. time (Text-fig. 2); and on the average, blebs starting at 10.5 mm are 19.5 mm in diameter after 2 hr and 20.5 mm after 4 hr. The rate of spread is much the same in freshly killed animals. The decrease in thickness is partly due to seepage through the muscle layer into the subcutaneous tissue, which becomes noticeably wet; and partly to the outward spread of the fluid. Of the forces responsible for the outward spread, diffusion is unlikely to play any large part, because the intradermal invasion of diffusible substances applied to the cut edge of normal skin is small and slow (e.g. the enzyme hyaluronidase, Hechter, 1946). Spread probably takes place by mass movement of the fluid either by reason of the hydrostatic pressures engendered in the tissue during injection or because the tissue has an affinity for water, which moves therefore from the hydrated bleb to the surrounding less hydrated tissue.



Text-fig. 2. Growth of an intradermal bleb in the guinea-pig formed by 0.1 ml. saline.  
Each point the mean of three blebs.

Large syringe pressures are required to initiate an intradermal bleb. In a series of measurements on ten guinea-pigs, this pressure varied between 80 and 140 cm Hg; once the bleb was begun, it increased rapidly in size under syringe pressures of 60-100 cm Hg. Only 1-2 cm Hg were required to expel fluid rapidly from the unimpeded syringe needle. The tissues do not, however, store the energy to a degree represented by these pressures, because when the bleb is cut vertically across its middle, fluid oozes only very slowly from the cut surface. Indeed, the pressure within the bleb soon after it is made cannot be higher than that in the small vessels involved, because exudation from the vessels takes place in blebs only 3 min old (see p. 236). At this time the maximum pressure must therefore be less than that in the largest vessels from which exudation takes place; and probably does not exceed 1-2 cm Hg. That the occlusion of the vessels during injection is only short-lived can be seen when

the formation of an intradermal bleb is watched under low-power magnification in a transilluminated hyperaemic ear. The hyperaemia is apparently restored after a few seconds. In arteries 0.4–0.8 mm in diameter, the flow returns in 5–10 sec; and in veins 0.5–2 mm in diameter flow returns in 30–120 sec, and initial diameter is restored in under 3 min. The high injection pressures are apparently needed only to tear apart the tissues at the advancing edge of the bleb.

Fluid may be expressed more rapidly by gently pinching the cut edge of a bleb between finger and thumb. A great deal of the fluid therefore cannot be held by hydration of the tissues. It is presumably held in innumerable small, distended, interconnecting loculi in the connective tissues, and is forced outwards to the periphery of the bleb by contraction of the distended tissue fibres. Hydration, however, may well account for the retention of some of the fluid in a cut bleb, as the following experiment shows. The average water content of pieces of muscle-free, intact skin from the trunk was about 50%; i.e. one part of dry matter holds about one part of water. Pieces of fresh guinea-pig skin were cut into 10  $\mu$  slices on a freezing microtome, washed in saline to remove damaged cells and the contents of the cells cut open during section, and the washed slices allowed to imbibe water from 0.85% saline for 30 min at 32° C. They were then deposited as a hard cake by centrifugation, and excess fluid removed from the deposit by firm pressing between sheets of filter-paper. The average water content of these masses was about 70%, i.e. one part of dry matter can hold 70/30 = 2.3 parts water. A 0.1 ml. bleb occupies about 160 mg of intact skin, which, if it attained the same degree of hydration as the sliced skin, could hold  $80 \times 2.3 = 184$  mg water; i.e. not only the 80 mg of natural water, but the injected 100 mg as well.

#### *The dosage-response to intradermally injected substances*

The distribution in the skin of an intradermally injected substance will depend on its concentration and the rate at which it is adsorbed or 'fixed' by the tissues during the outward flow of injection fluid from the needle. A substance that was adsorbed very little would spread with the injection fluid, and, according to the evidence in Text-fig. 2, would eventually produce lesions whose diameter was directly proportional to the diameter of the injection bleb; i.e. to the volume of fluid injected. Most substances, however, are adsorbed in some degree, and accumulate at and around the centre of the bleb. The relation of the bleb-diameter to lesion-diameter with different drugs can be used to characterize their adsorption to the tissues. It was explored for histamine, 48/80 and leukotaxine by two methods of injection in blued animals: (a) graded concentrations in a constant injection-volume, and (b) graded injection-volumes containing a constant dose.

'Constant-volume' measurements. Four doses of the substance under test in

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## VASCULAR REACTIONS TO HISTAMINE

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0.1 ml., were randomized in a 4 × 4 Latin square on the trunk, two rows on each side of the spinal mid-line. Histamine, 48/80 and leukotaxine all produced round blue lesions in the skin and in each case the lesion-diameters measured 30 min after injection were linearly related to the log. dose (cf. Text-fig. 5a, b). The responses were subjected to analysis of variance, with the results exemplified in Table 1, which records a titration of histamine in three blued animals. The departure from linearity of the regression line of lesion-diameter on log. dose is insignificant. The variation between columns and rows is also insignificant, so that for practical purposes the skin of the more dorsal

TABLE 1. Analysis of variance of a titration of histamine in a 4-fold Latin square in three blued guinea-pigs

	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P
Between animals	2	3.2279	1.61395	1.66	>0.05
Between columns	3	3.1275	1.0425	1.07	>0.05
Between rows	3	2.0174	0.6725	—	—
Between doses	3	312.7425	104.2475	107.46	<0.001
Linearity	1	72.8017	72.8017	75.05	<0.001
Curvature	1	3.2939	3.2939	3.40	>0.05
Response	1	235.8784	235.8784	243.15	<0.001
Error	36	34.9239	0.9701	—	—
Total	47	356.0392	—	—	—

part of the guinea-pig's trunk may be considered homogeneous in its sensitivity to histamine. (This is not true of the thinner ventral skin, where lesions tend to be larger.) Each animal yields a mean of four responses per dose. The error term for inter-animal variation is large, but reliable comparisons between various treatments were obtained in other tests by using four to six animals per group. This linear relationship differs from that found by Bain (1949) in the human skin, where area of histamine whealing was proportional to log. dose. Linearity of diameter against log. dose holds for many substances in guinea-pig skin: diphtheria toxin (Miles, 1949), tuberculin in tuberculous animals (Wadley, 1949) and appears to hold for other bacterial antigens in hypersensitive animals, and for the lesions produced in the blued animals by various toxins such as the exotoxins of *Clostridium welchii*, *Cl. oedematiens* and *Staphylococcus aureus*, and for cobra venom (unpublished work). The difference in Bain's titration may lie in the modification of histamine spread or of wheal-diameter, by the copious exudation during whealing that occurs in man but not in the guinea-pig.

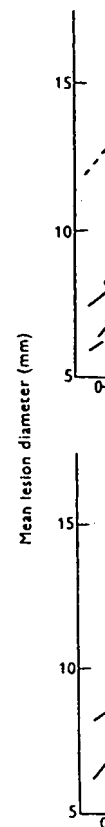
Full 4 × 4 Latin-square titrations were usually made only when the significance of a result was in doubt; in most of the tests fewer replications of doses were used, partially randomized among three to six animals per group. In these titrations, 48/80 and leukotaxine differed from histamine mainly in the slope of the dosage-response lines. Slopes varied with each experiment, and particularly with the amount of dye injected. Moreover, since the concentration of circulating dye falls rapidly during the first 2 hr, slope decreases with lapse of

TESTED  
Placement  
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time between blueing the animal and the intradermal titration. In guinea-pigs receiving 65 mg/kg tested soon after its injection, the slopes for histamine lay between 2.8 and 3.5, for 48/80 between 1.5 and 2.0 and for leukotaxine between 1.5 and 4.0. The shallow slope of 48/80, which indicates an increase of only 1.5–2.0 mm in lesion-diameter for a 10-fold increase in concentration, suggests that, compared with histamine, it is strongly adsorbed to the tissues. It should be noted that the susceptibility of this linear dosage-response to statistical analysis makes possible an accurate though not very precise measure of the potency of each of the three substances. The method is too insensitive for routine assay, but was well adapted to our investigations of skin reactions. Thus where parallel regression lines can be fitted to two sets of log. dose-diameter responses, the horizontal distance between the two lines is the log. ratio of drug potency; and, for a given specimen of a drug, it is the log. inverse ratio of sensitivity to the drug. For example, in Text-fig. 5*b*, 3 mg neoantergan has shifted the slope to the right by 0.43 = antilog. 2.7. That is, the neoantergan has decreased the 48/80-sensitivity of the animals 2.7-fold, since 2.7 × the dose in normal animals is required to produce the same effect in the treated animal.

'Constant-amount' measurements. In this method a fixed amount of the drug was injected in volumes of 0.05, 0.1, 0.2 and 0.4 ml.; i.e. the concentration of the drug is varied. The diameter of the blebs, measured immediately after the injections, was linear with respect to log. volume (Text-fig. 3*a*, *A*); and, according to the data in Text-fig. 2, after a given period (e.g. 30 min, Text-fig. 3*a*, *B*) the expanded bleb-diameters would be linear, on a line parallel to that for the immediate bleb-diameters (Text-fig. 3*a*, *B*). The slope of the initial bleb-diameters, irrespective of any lesion produced by the drug injected, is relatively constant in the region of 9.0. When the drug has acted the resulting lesion-diameters are also linear with respect to log. injection volume. Text-fig. 3*a*, *C* and *D*, records results with 12.5 and 50 µg pontamine blue in unblued animals. The 'lesion' here is the area of skin coloured by the injected dye. The slope of lesion-diameter is not parallel to that for initial bleb-diameter, which would have indicated no adsorption during injection. Nor is it horizontal, which would have indicated an adsorption so strong that it was independent of concentration within the range tested. The slope of *C* and *D* is in fact 5.5, compared with 9.7 for the initial bleb-diameter. Pontamine blue has a good affinity for skin tissue (Evans, Miles & Niven, 1948), and we would therefore expect slopes like *C* and *D*, which show that as the solutions are forced outwards during injection, adsorption decreases with decreasing concentration of the injection fluid. The magnitude of the slope, compared with that for the initial bleb, is a reasonable inverse indicator of the affinity of the tissues for the drug. Text-fig. 3*b–d*, and Table 2 summarize similar titrations on histamine, 48/80 and leukotaxine; the slopes indicate that the tissue affinity is least for histamine, and greatest for leukotaxine.

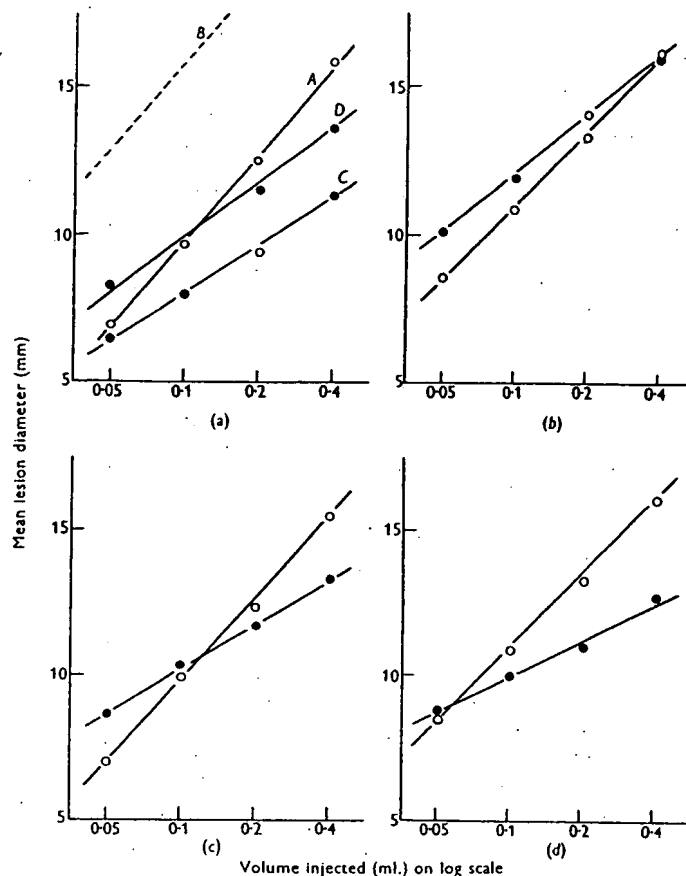
V<sub>2</sub>  
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bleb from 0.1 ml



Text-fig. 3. Constant amount injected. Each diameter of the bleb by the substance after 30 min. (a) 12.5 µg dye. (b) 50 µg dye. (see pp. 234–6.)

to fill the 0.2 ml spreads outward dye is carried so dye has spread c initial bleb-dian

The relative position of the two slopes is also informative. In Text-fig. 3*a*, the 50  $\mu$ g line *D* crosses *A* at about 10 mm, i.e. the skin occupied by the initial bleb from 0.1 ml. just adsorbs the dye. The 50  $\mu$ g of dye is clearly insufficient



Text-fig. 3. Constant-dose intradermal titrations. Lesion-diameters plotted against log. volume injected. Each point the mean of twelve to sixteen lesions. In all the graphs,  $\bigcirc$  —  $\bigcirc$ , mean diameter of the initial blebs raised by the fluid injected;  $\bullet$  —  $\bullet$ , diameter of lesion produced by the substances injected. (a) pontamine blue: *A*, initial bleb-diameter; *B*, bleb-diameter after 30 min calculated from data in Text-fig. 2. *C* and *D*, lesion-diameters of 12.5 and 50  $\mu$ g dye. (b) Histamine, 8  $\mu$ g; (c) 48/80, 13  $\mu$ g; and (d) leukotaxine, 40  $\mu$ g, in blued animals (see pp. 234–6 and Table 2).

to fill the 0.2 ml. bleb. It oversaturates the 0.05 ml. bleb so that as the fluid spreads outwards from the region initially filled by the injection, the excess of dye is carried some way with it; at 30 min, when the reading was taken, the dye has spread over 8 mm and the fluid (line *B*) over 12.5 mm. The intersect of initial bleb-diameters and lesion-diameters is thus a convenient measure of the

tration. In guinea-slopes for histamine and for leukotaxine indicates an increase of e in concentration, rbed to the tissues. dosage-response to ery precise measure d is too insensitive as of skin reactions. sets of log. dose-wo lines is the log. it is the log. inverse , 3 mg neoantergan is, the neoantergan since  $2.7 \times$  the dose the treated animal. amount of the drug e concentration of immediately after t-fig. 3*a*, *A*); and, e.g. 30 min, Text-line parallel to that of the initial bleb-ected, is relatively e resulting lesion-ume. Text-fig. 3*a*, a unblued animals. l dye. The slope of eter, which would horizontal, which dependent of con-fact 5.5, compared a good affinity for fore expect slopes outwards during n of the injection the initial bleb, is or the drug. Text-amine, 48/80 and ast for histamine,

adsorbing power of the skin for a given dose of drug, though clearly it can give no information about the concentration gradient of the drug within the bleb. It is the volume in which the adsorption of the drug is so balanced that the minimal effective concentration is produced at the edge of the initial bleb; and may be called the critical volume. In Text-fig. 3a, the critical volume for 50  $\mu$ g of pontamine blue is 0.105 ml.

TABLE 2. Slopes and critical volumes in 'constant-amount' titrations of histamine, 48/80 and leukotaxine

Substance	Approx. equimolar dose ( $\mu$ g)	Slope mean diameter		Critical volume (ml.)
		Initial bleb	Lesion	
Histamine	8	8.6	6.6	0.400
48/80	13	9.3	5.2	0.012
Leukotaxine	40	8.6	4.1	0.058
(Pontamine blue)	—	9.8	6.2	0.105)

For a valid comparison, the critical volumes of equimolar concentrations of histamine, 48/80 and leukotaxine were determined from the data in Text-fig. 3c-e (Table 2). 8  $\mu$ g of histamine was tested, and the approximate molecular equivalent, 13  $\mu$ g, of 48/80. The result for an equimolar amount of leukotaxine (40  $\mu$ g) was extrapolated from the slope for 20  $\mu$ g using the value 4.0 for the slope of a constant-volume titration. The values both for slope and critical volume must be regarded as very approximate. Histamine (Table 2) has a high 'constant-amount' slope, and a high critical volume; it is thus relatively poorly adsorbed during injection, and the skin is relatively poor in adsorbing sites. Both 48/80 and leukotaxine are more strongly adsorbed, and the skin is richer in adsorbing sites.

#### *Skin reactions to histamine*

Histamine even as strong as 1.6% will not induce blueing in dyed animals when applied to undamaged skin. When the skin of blued animals is damaged by rubbing (cf. Matolsty & Matolsty, 1951) or scratching sufficient to produce patches of traumatic blueing, the application of 1% histamine will increase the size and intensity of such blueing, presumably because the drug, having penetrated the damaged skin, diffuses inwards from the damaged area. It is also possible to induce blueing by the electrophoresis of 1% histamine; the histamine is not however driven in uniformly, but produces irregular patches of blueing. In none of these tests was whealing ever observed.

*Intradermal injection in the trunk.* Histamine injected intradermally into blued animals produces a round area of blueing that appears in 3-5 min and increases slightly in diameter and intensity during the next 7 min. In a volume of 0.1 ml., as little as 0.1-0.2  $\mu$ g is effective; with increasing dose the blue increases in intensity and area. With amounts greater than 1-3  $\mu$ g the colour first appears at the edge of the bleb, but gradually fills the centre. With doses

of 4  $\mu$ g and blueing round than 10-20 the bleb.

On section stained at the feebly stained region of the (Text-fig. 1c) there is blue and thoracic the central colourless zone.

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## VASCULAR REACTIONS TO HISTAMINE

of 4  $\mu$ g and more, the centre remains uncoloured except for the traumatic blueing round the site of needle-entry, and colour develops at the edge. More than 10-20  $\mu$ g produces only a narrow band of very faint blue at the edge of the bleb.

On section after 10 min, a 1  $\mu$ g histamine lesion is seen to be uniformly stained at the skin surface, more widely stained in layer *B* (Text-fig. 1c), and feebly stained in the muscle. Occasionally the staining is slightly deeper in the region of the two plexuses of blood vessels. With stronger doses of histamine (Text-fig. 1d), the drug has in 10 min spread downwards and outwards so that there is blue exudate in the underlying subcutaneous tissues, and the abdominal and thoracic muscles may be stained. The other feature of the stronger dose, the central inhibition of blueing, is also evident in cross-section, where the colourless zone reaches down to the muscle layer.

The linear response, lesion diameter on log. dose, holds with histamine for diameters between 5 and 17 mm: below 4 mm histamine blueing may be confused with traumatic blueing. In reading the diameters, inhibition of blueing at the centre is ignored even though with the higher concentrations of the drug only faint, thin rings of blue may be produced. The lesion-diameters measured on the under-surface of flayed skin are larger than those on the upper surface, by about 40%. This ratio is approximately constant. There is, however, no advantage in the measurement, because the lesions are less well defined and, with the bigger doses, obscured by subcutaneous blue exudate.

*Intradermal injections in the ear.* Blueing of the ear, as in the trunk (see below), was partly inhibited by bromethol anaesthesia; but under light anaesthesia it was sufficiently strong for a number of useful observations, though lesion diameters were not such a consistent guide to drug-sensitivity. The ear was found to be less sensitive to histamine than the skin of the trunk. In unblued animals, concentrations of 5-1000  $\mu$ g/ml. caused immediate flushing of the skin of the bleb, which lasted about 10 min. Stronger histamine, up to 10,000  $\mu$ g/ml. also caused immediate flush, and after 1½ min an intense vasoconstriction, lasting 3-5 min, of the arteries traversing the bleb area.

In blued animals, the permeability effect is visible in 1½ min. The minimal blueing concentration was about 0.5  $\mu$ g/ml. Central inhibition of blueing occurred at 50  $\mu$ g/ml., lasting only 3 min, after which the centre of the bleb became blue. At 1000  $\mu$ g/ml. it lasted about 16 min, unlike the central inhibition by 50  $\mu$ g/ml. in the skin of the trunk, which persisted for several hours. The results in Table 6 were obtained from blebs of 3 mm initial diameter; the injection volume was not measured exactly.

With concentrations of 100  $\mu$ g/ml. the area of blueing of a 3 mm bleb was 10 mm in diameter after 10 min. Histamine in stronger concentrations, 1000-10,000  $\mu$ g, leaked into the lymphatic plexus, spread both distally and proximally through the freely anastomosing channels, passing back into the

tissues to produce an oedematous wedge-shaped area of intense blueing with its apex at the base of the ear where the main lymphatic ducts leave the ear (Pl. 1A, B). This is a characteristic lymphatic spread of strong histamine in the ear, and is also produced by strong histamine injected directly into the lymphatic plexus. It is evident therefore, that histamine readily passes both ways through lymphatic endothelium.

*Factors that change skin-reactivity to histamine in the trunk*

**Temperature.** Depilated animals held at 10° C react poorly to histamine; those at 20° C react well; and those at 37° C poorly and irregularly. Thus, the mean lesion-diameters for 3, 9 and 27 µg histamine were 6.7, 8.3 and 9.6 mm at 20° C, and 4.7, 5.7 and 5.9 mm in animals held at 37° C. The intensity of blueing was considerably less at 37° C. In this reaction to heat the guinea-pig is similar to man in his whealing reaction to histamine (Lewis & Grant, 1924).

**Anaesthesia.** Under ether, chloroform, chloralose, bromethol, pentobarbitone and urethane anaesthesia, blueing is greatly diminished or even abolished. In general, the deeper the anaesthesia, the greater the inhibition of blueing. After a short period of anaesthesia with ether, bromethol or chloroform, reactivity is sometimes restored on recovery. Loss of reactivity is accompanied by a decline in the pressure of the central arteries of the ear, measured by a modified Grant's capsule (Miles & Niven, 1950), from 40 to 70 mm in the unanaesthetized state, to 20–40 mm Hg. This suggests that in the skin of the trunk also there is in anaesthesia a decline in blood pressure to the point where dye is no longer forced out into the tissues, though the permeability of the vessels may be increased by the histamine. However, in many recovered animals, in which the blood pressures in the ear have returned to normal, the skin of the trunk remains unresponsive; either the anaesthetic has modified histamine-sensitivity, or the circulation is in these areas restored less quickly than in the ear.

**Shock.** In guinea-pigs given sublethal shocking doses of *Proteus vulgaris* and *Bacterium coli* endotoxins, of adenosine-triphosphate, insulin or intraperitoneal hypertonic glucose, reactivity to histamine is diminished; and, as in anaesthesia, the loss is associated with low blood pressure in the ear, and with low skin temperature (Miles & Niven, 1950). In shocked or anaesthetized blued animals, whose skin does not respond to histamine, it is possible to demonstrate an increase in capillary permeability by indirectly raising the intracapillary pressure. When a suction cup is applied to the skin of these animals, substantial blueing of a histamine-treated area is produced within 5 min, by a suction of 10 mm Hg applied intermittently for 2–3 sec every 10 sec.

**Infection and malnutrition.** Guinea-pigs suffering from spontaneous chronic infective abscesses, or in poor health because of heavy infection with B.C.G.

(*Bacillus of Calmette and Guérin*) of vitamin C, reactivity to histamine is diminished.

**Cortisone, pituitary lobe extract and ACTH.** Cortisone, pituitary lobe extract and ACTH, when administered in doses of 10–20 mg of cortisone and 10–20 units of ACTH, diminish the intensity of blueing in the guinea-pig (Loewen, 1950). Cortisone had no effect on the lesions of the trunk, but the staining was greatly diminished by the time, the skin of the animals was slightly blue. Near normal reactivity to ACTH and cortisone extractives, administered in doses of 10–20 units of ACTH and 10–20 mg of cortisone, given subcutaneously, was observed. Certain P.P.E., given subcutaneously, had no effect on the titration, but histamine (Texter, 1950) the diminution of blood supply, but not more than that of the controls.

**Neoantergan.** Neoantergan, in 0.1 ml. itself produced a tense blueing of the skin. Histamine gave a tense blueing, and neoantergan is an antihistamine, on the skin. Histamine blueing of the skin about the trunk.

Excepting in the guinea-pig, by intravenous administration can be attributed to histamine sensitivity to histamine, due, as Lewis & Grant (1924) rapid removal of histamine from the variety of the skin.

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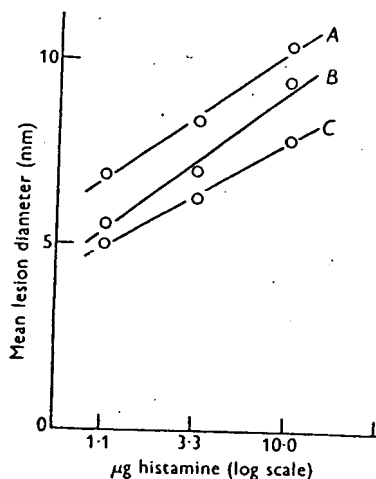
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(Bacillus of Calmette and Guérin) or as a result of long-term partial deficiency of vitamin C, react poorly to histamine.

*Cortisone, preparations of adrenocorticotrophic hormone (ACTH) and posterior pituitary lobe extract (P.P.E.).* In an attempt to relate the anti-allergic effect of cortisone and ACTH to an effect on histamine sensitivity, histamine was titrated in blued animals, 2-3 hr after doses of cortisone (2 mg) or ACTH (1 i.u.). These doses had proved effective in diminishing tuberculin allergy in the guinea-pig (Long & Miles, 1950). The cortisone had no effect. The diameters of the lesions were not substantially changed by the ACTH, but intensity of staining was greatly diminished. At this time, the skin of the ACTH-treated animals was slightly colder than those of controls. Nearly all current preparations of ACTH contain some posterior lobe extractives, and these may have been responsible for the ACTH effect we observed. Certainly both 2 and 0.2 i.u. P.P.E., given subcutaneously 2½ hr before the titration, diminished reactivity to histamine (Text-fig. 4); and here again the diminution was probably due to poor blood supply, because the skin was cooler than that of the controls.

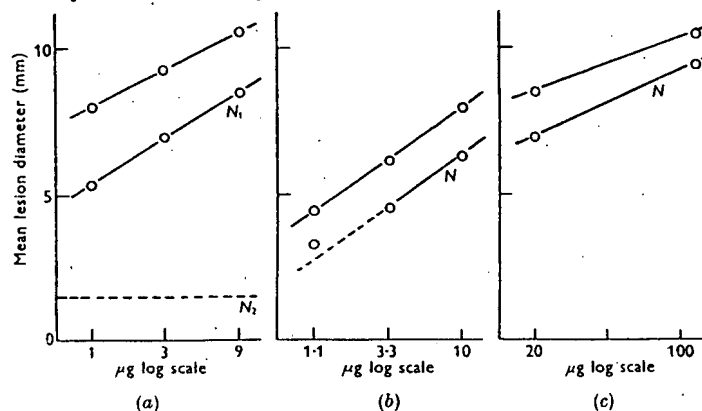
*Neoantergan.* 20 µg neoantergan (mepyramine maleate) given intradermally in 0.1 ml. itself induced slight blueing of the skin; 5 µg histamine induced intense blueing 11 mm in diameter; and 20 µg neoantergan mixed with 5 µg histamine gave a pale blue area 10 mm in diameter. Local neutralization by neoantergan is therefore possible, but not very effective. Intravenous neoantergan, on the other hand, is most effective; 0.1 mg/kg almost abolished histamine blueing, and 0.02 mg/kg diminished the efficacy of intradermal histamine about 9-fold (Text-fig. 5a).

Excepting inhibition in animals held at high atmospheric temperatures, and by intravenous neoantergan, most of the effects on blueing described above can be attributed to a decline of intravascular pressure, rather than to insensitivity to histamine. The insensitivity of an abnormally warm skin may be due, as Lewis & Grant (1924) suggested for the human subject, to the more rapid removal of histamine in the more physiologically active tissue. The variety of the states in which there is inhibition of blueing is a warning that



Text-fig. 4. The depressant effect of intramuscular posterior pituitary extract (P.P.E.) on histamine blueing in the guinea-pig. Each point the mean of twelve lesions. A, untreated animals; B, 0.2 i.u. P.P.E., 2.5 hr earlier; C, 2.0 i.u. P.P.E., 2.5 hr earlier.

absence of local blueing cannot safely be interpreted as absence of increase in capillary permeability unless there is good reason to believe that the blood supply to the skin and the state of the skin vessels have not been altered by the experimental procedure. On the other hand, it is reasonable to assume that a substance like histamine, which rapidly induces a deep blueing in healthy animals held at an atmospheric temperature of about 20° C, does so by an abnormal increase in capillary permeability. Histamine can act as vasodilator, but it is unlikely that the increased blueing in the guinea-pig is due to increased flow and exudation of dye as a result simply of vasodilatation, as suggested by Dekanski (1949). The rate of histamine blueing is too rapid to be attributable to this cause. Histamine induces in 3 min an intensity of blueing reached by untreated skin in 10 hr or more; i.e. the rate of accumulation of the dye is increased by over 200-fold.



Text-fig. 5. The effect of neoantergan ( $N$ ) on blueing in the guinea-pig. Each point the mean of twelve or sixteen lesions. (a) Histamine,  $N_1 = 0.02$  mg/kg,  $N_2$  = average diameter of lesion with 0.1 mg/kg; (b) 48/80,  $N = 8$  mg/kg; (c) leukotaxine,  $N = 3$  mg/kg.

#### The time-course of the histamine effect

*The rate of 'fixation' of histamine.* The 'constant-amount' titration of histamine (Text-fig. 3b, Table 2), measuring the blue area developing in 10 min, shows that during injection the drug is lightly adsorbed. Within 5 min, however, some reaction with tissue must have occurred, because the increased permeability is by that time almost fully developed. The rate of that reaction may be estimated by the technique of superinjection (Miles, 1949) in which a substance is injected through a needle painted with a trace of indian ink so that the site of needle entry is exactly marked; and after an interval an injection of saline made into the same site. If any of the injected substance is free, it will be displaced outwards by the superinjected saline beyond the periphery of the initial bleb, with a consequent increase in the size of the lesion; if there is no increase, the injected substance has already been held fixed or destroyed by the tissues.

In applying the method from increase in lesion size, saline may push, not its original confines. the rate at which the serum was coloured stained the skin to the extent of a blue animal. superinjected after indicated that some of the dye could be dislodged by

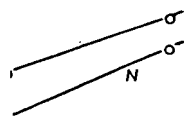
Text-fig. 6. The fixation of dye by 0.2 ml. saline. C =

hoped for from the steady value, but the lesion was left to develop with the blebs made in blueing in 3-4 min, a period starts, the greater 16 min old lesions. all the histamine might be expected, is taking place.

*Duration of increase in lesion size.* measured by injecting saline into the animal, and giving dermal injection; the dye is injected,



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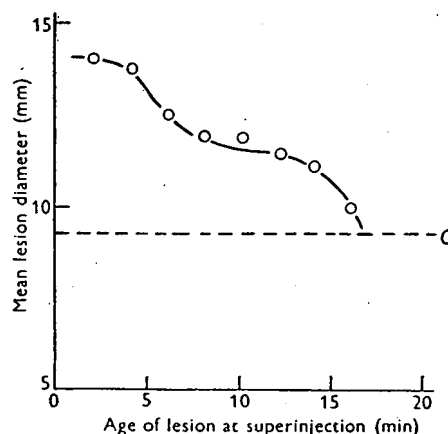
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## VASCULAR REACTIONS TO HISTAMINE

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In applying the method to histamine and histamine-liberators the evidence from increase in lesion-diameter is not unambiguous because the superinjected saline may push, not free histamine, but already-formed blue exudate, beyond its original confines. The ambiguity may be resolved in part by determining the rate at which 'exudate' is fixed to the tissues. To this end, guinea-pig serum was coloured with pontamine blue so that in an unblued animal 0.1 ml. stained the skin to the intensity produced by 3 μg histamine in 0.1 ml. injected into a blued animal. Into the sites of injection of this fluid, 0.2 ml. saline were superinjected after graded intervals of time. The results were variable but indicated that some of the dyed serum was fixed within 3-4 min and the rest could be dislodged by superinjection up to 20 min. later. The best that can be

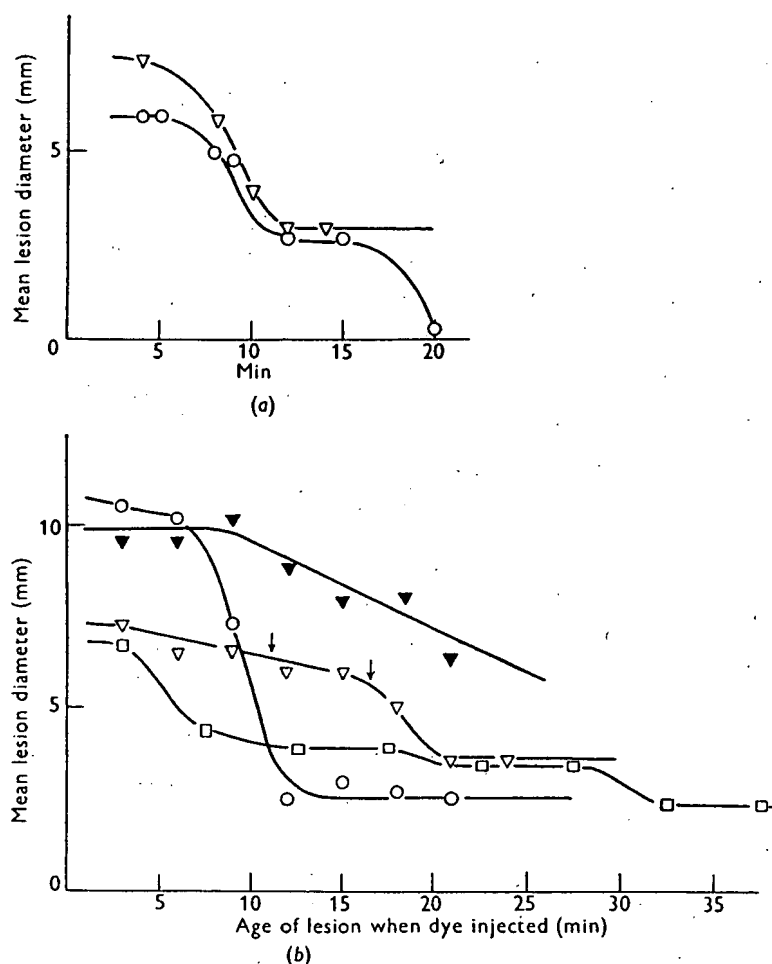


Text-fig. 6. The fixation of 3 μg histamine by guinea-pig skin. Superinjection of histamine lesions by 0.2 ml. saline. C = average diameter of control lesions. Each point the mean of four lesions.

hoped for from the test is that with time the lesion-diameters decline to a steady value, but this will not necessarily be the same as the diameter of lesions left to develop without superinjection. When saline was superinjected into blebs made in blued animals with 3 μg histamine (Text-fig. 6) little was fixed in 3-4 min, a period corresponding to the latent period before blue exudation starts, the greater part was fixed in 4-12 min, and nearly all was fixed in 16 min old lesions. If we allow 3 min for the fixation of the blue exudate, then all the histamine may be fixed in as little as 13 min, and most of it is fixed, as might be expected, during the period when the histamine-induced exudation is taking place.

*Duration of increased permeability.* The duration of the histamine effect was measured by injecting 2 μg in 0.1 ml. at regular intervals for 30 min in an animal, and giving the dye intravenously immediately after the last intradermal injection; there are thus lesions varying in age from 1 to 30 min when the dye is injected, and lesions in which the vessels are no longer permeable do

not blue. It is clear from Text-fig. 7*b* that after 10 min the capillaries have recovered; only the traumatic blueing caused by the needle remains. A similar result was obtained in the ear (Text-fig. 7*a*). Here the residual traumatic blueing due to the fine micro-needle is minimal.



Text-fig. 7. The duration of increased permeability in guinea-pig skin. In each case there is a decline to the level of traumatic blueing induced by the injection needle. (a) The ear: histamine, approx. 2  $\mu$ g in 0.02 ml., ○—○; 48/80, 2  $\mu$ g in 0.02 ml., ▽—▽. (b) The trunk: histamine, 1  $\mu$ g in 0.1 ml., ○—○; 48/80, 20  $\mu$ g in 0.1 ml., ▽—▽; 2  $\mu$ g in 0.1 ml., ▽—▽; leukotaxine, 10  $\mu$ g in 0.1 ml., □—□.

*Immunity to histamine.* It follows that the site of a histamine injection remains colourless if it is 10–15 min old when dye is given. When more histamine is superinjected into such a site, blueing is either feeble or absent. The

injection site has immunity is not a form of diminished immunization; or of the blood vessel reaction. This irregular terms of lesion-diminished tissues are usual of the change.

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# VASCULAR REACTIONS TO HISTAMINE

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injection site has become refractory—or immune—to further histamine. The immunity is neither solid nor regular in its manifestation; it may take the form of diminished area of feeble staining, as though all the tissue were partly immunized; or of small patches of blue from 1 to 4 mm in diameter, as though the blood vessels in certain areas of the injection site had escaped immunization. This irregularity of response partly spoils measurement of immunity in terms of lesion-diameter, but the differences between immune and non-immune tissues are usually so large that mean lesion-diameters are good enough indices of the change. It is clear, for example, from Table 3, that after 1 hr 9  $\mu$ g

TABLE 3. Immunizing action of intradermal histamine to injections of histamine made 1 hr later. Means of three lesions

Immunizing dose of histamine ( $\mu$ g)	Mean diameter (mm) and intensity* of reaction to test dose of histamine		
	1 $\mu$ g	3 $\mu$ g	9 $\mu$ g
Nil	8.5 + +	10 + +	11.3 + +
9	0	6 f.	7.8 f.

\* f.,  $\pm$ , +, + + = faint, moderate, marked, intense blueing.

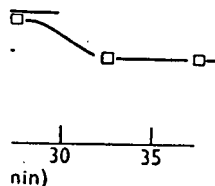
induces a solid immunity to 1  $\mu$ g, and a substantial immunity to 9  $\mu$ g. A number of similar experiments established that though some immunity is present after 10 min, when the capillaries have recovered from the immunizing dose, it is greatest in lesions 1½–2½ hr old, and is passing off after 4 hr.

Histamine in concentrations of 10–100  $\mu$ g/ml. immunized the ear vessels to histamine. Immunity was irregular in lesions up to 35 min old, well established after 40 min, maximal at 2–3 hr and lasted up to 5 hr. After 40 min, 50  $\mu$ g/ml. had immunized to a test dose of 50  $\mu$ g/ml. so that the diameter and intensity of blueing was reduced from 9 mm + +, in a control area to 4 mm  $\pm$ , in the immunized area; 20  $\mu$ g/ml. did not immunize to 50  $\mu$ g/ml., and the immunity induced by 10  $\mu$ g/ml., though it protected against 10  $\mu$ g/ml., lasted only 60 min.

## The inhibition of skin-reactivity by high concentrations of histamine

The restriction of blueing to the periphery of blebs containing 4  $\mu$ g histamine or more may be explained in terms of the recovery of normal impermeability and of immunity, if we also postulate that the histamine at the centre of the bleb was strong enough to induce vasoconstriction of the arterioles for at least 10 min. As a result, the centre of the bleb would not blue during this time because the blood supply is cut off—a state corresponding to the inhibition of whealing in man by pressure-occlusion of the vessels—and by the time the blood supply is re-established, the central capillaries would have recovered from the histamine. Alternatively, the reaction of the vessels with high concentrations of histamine may proceed so rapidly that the immune stage is reached before the preceding stage of permeability can manifest itself by the escape of dye.

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Lewis & Grant (1924) inferred that the refractory state was not due to occlusion of the lumen of the vessels by viscous R.B.C. or other material, because it could be induced in a relatively bloodless arm, and because, even though whealing was inhibited, vascular reactions that indicated a fully patent vascular bed could still be elicited. There is no anatomically convenient site in the guinea-pig for occlusion tests as applied by Lewis & Grant, and the dermis is too dense for direct observation of vascular changes. The vessels were proved patent by direct test. Injections of 5 or 10  $\mu$ g histamine were made at intervals, in the skin over the right scapular region of normal guinea-pigs under urethane anaesthesia. At the end of the series of skin injections, 4 ml. indian ink was put into the right axillary artery. During the preparation of this artery, the blood supply to the skin was interrupted for the last 2-3 min of the experiment. The state of the vascular bed was deduced (a) by direct observation of the blackening of the skin; (b) from the pattern of the ink-filled vessels seen by low-power microscopy in excised skin areas fixed in 10% formalin, dehydrated and cleared with clove oil; and (c) from the distribution of ink in the lumen of capillaries seen in histological section of individual blebs. In tests by these methods on eight animals, there was no evidence of blocked vessels in histamine lesions from 5 to 30 min old.

#### *Skin reactions to 48/80*

*Intradermal injection in the trunk.* In the skin of blue animals, 48/80, like histamine, induces a round area of blueing whose diameter is linearly related to the log. dose injected in a constant volume of 0.1 ml. (Text-Fig. 5b). The slope of the dose-response line is much less than that of histamine, varying between 1.5 and 2.0. The blueing develops in 3-5 min and is more intense than that produced by histamine; the minimal effective dose distinguishable from traumatic blueing by the injection needle is about 0.2  $\mu$ g. With doses of 2-4  $\mu$ g the blue first appears at the periphery of the bleb and invades the centre. In cross-section of 48/80 lesions the blue area is confined to the two upper layers of the skin (Text-fig. 1e). The blueing is not uniform as it is with histamine; it is more intense in the region of the two plexuses of blood vessels, and especially that immediately over the muscle layer. The 48/80 does not spread downwards to the subcutaneous tissues. Even with doses of 30  $\mu$ g in 0.1 ml., only the upper part of the muscle layer is blue, and there is no blueing of subcutaneous tissues or underlying muscle. This is not due to absence of histamine available for liberation in the muscle or subcutaneous tissues, because direct injection of 48/80 into these sites induced intense localized blueing. This difference in the spread of 48/80 and histamine from the injection site is not likely to be due to the difference in molecular weights, which are of the order of 540 for the trimer of 48/80 and 310 for the acid phosphate of histamine; the difference confirms the conclusions about the greater adsorption of 48/80 derived from the results of the 'constant-amount' titrations (Text-fig. 3c and Table 2).

The centre of the it becomes pink with of haemorrhage (Temporary vasoconstriction followed by recovery. But it is to a large thrombotic, to the surrounding skin is blocking of the vessels.

*Intradermal injection.* An immediate flush 100  $\mu$ g/ml. in 1½ min treated area, where 5 min, along the centre under it. These par lesion. Above 250  $\mu$ . There is a central area within 1½ min, the tion. The underlying blood after about central area, so that surrounded by deeper persist for over 6 h.

Though 48/80 spreads from initial bleb, the result at 10,000  $\mu$ g/ml. does the bleb to produce directly into the periphery 15 min or more after but this is observed from the bleb; it adjacent to the conclusions from that it is fixed firmly and freely, and begins centre is due to the as fast as histamine effect is almost identical that 48/80 liberates of the liberated histamine.

*Factors that characterize* by histamine, is

state was not due to other material, because and because, even though a fully patent vascular convenient site in the Grant, and the dermis is. The vessels were proved ine were made at inter-ormal guinea-pigs under injections, 4 ml. indian the preparation of this or the last 2-3 min of the d (a) by direct observa- of the ink-filled vessels fixed in 10% formalin, he distribution of ink in individual blebs. In tests lence of blocked vessels

ed animals, 48/80, like neter is linearly related nl. (Text-Fig. 5b). The of histamine, varying nd is more intense than se distinguishable from g. With doses of 2-4  $\mu$ g invades the centre. In o the two upper layers it is with histamine; it vessels, and especially not spread downwards 0.1 ml., only the upper eing of subcutaneous of histamine available use direct injection of This difference in the s not likely to be due e order of 540 for the amine; the difference of 48/80 derived from g. 3c and Table 2).

The centre of the 48/80 bleb remains unblued with doses over 5  $\mu$ g; at most it becomes pink with a purplish tinge, and sometimes there are small patches of haemorrhage (Text-fig. 1f). The absence of response may in part be due to temporary vasoconstriction by 48/80 itself or by the histamine it liberates, followed by recovery of normal permeability before the vessels become patent. But it is to a large extent due to a more permanent damage, presumably thrombotic, to the blood vessels, because the pink area remains when the surrounding skin is blackened by intra-arterial injections of indian ink; and the blocking of the vessels is evident in stained sections of the ink-treated skin.

*Intradermal injection in the ear.* Concentrations of 10 and 100  $\mu$ g/ml. caused an immediate flushing; with 10  $\mu$ g/ml. blueing started in 7 min, and with 100  $\mu$ g/ml. in 1½ min. Blueing in a histamine bleb develops uniformly over the treated area, whereas that due to 48/80 begins at the edge of the bleb and after 5 min, along the course of the large vessels, particularly the arteries, lying under it. These paravascular streaks then coalesce to form a uniformly blue lesion. Above 250  $\mu$ g/ml. this blueing occurs only at the periphery of the bleb. There is a central area whose size varies with the concentration, in which within 1½ min, the small superficial vessels thrombose without prior constriction. The underlying larger vessels become invisible but refill with circulating blood after about 10 min. By this time there is very slight blueing in the central area, so that by naked eye the lesion is faint purplish pink in the centre surrounded by deep blue (Pl. 2A). This central thrombosis and inhibition persist for over 6 hr.

Though 48/80 spreads outwards to blue an area 50-200 times that of the initial bleb, the resulting lesion is always round. Unlike histamine, 48/80, even at 10,000  $\mu$ g/ml. does not leak in the lymphatic plexus beyond the confines of the bleb to produce a wedge-shaped blue lesion. Even when 48/80 is injected directly into the plexus, the blue area is approximately round (Pl. 2B). After 15 min or more a blue prolongation towards the base of the ear may develop, but this is observably the coloration of the lymphatic ducts with blue exudate from the bleb; it is not due to an increased permeability of blood vessels adjacent to the channels, as with histamine. These observations confirm the conclusions from the behaviour of 48/80 lesions in the skin of the trunk, that it is fixed firmly and rapidly to the tissue, whereas histamine at first moves freely, and begins to be fixed only after 2-3 min; and that inhibition at the centre is due to thrombosis of the vessels. Moreover, since 48/80 lesions blue as fast as histamine lesions, but no faster, and since the duration of the 48/80 effect is almost identical with that of histamine (Text-fig. 7a), it is probable that 48/80 liberates histamine very quickly and its latent period is mainly that of the liberated histamine.

*Factors that change the skin-reactivity to 48/80.* Blueing by 48/80, like that by histamine, is partly inhibited during anaesthesia and shock, and by

warming the animals in an atmosphere at 37° C. Sickly animals respond poorly to 48/80; this is probably not due to a diminution in the skin of histamine available for liberation, because the same animals are proportionally insensitive to histamine itself. As with histamine, most of the states of apparent insensitivity to 48/80 appear to be due to a poor blood supply to the skin.

Neoantergan intravenously is much less effective with 48/80 than with histamine. The effect of 8 mg/kg body weight 30 min before the injection of 48/80 is shown in Text-fig. 5*b*. Three concentrations of 48/80 and a saline control were titrated in blue animals, the four doses being randomized in a Latin square over sixteen sites. Three animals were used in each group so that each point is the mean of twelve readings. There is a 3-fold decrease in the 48/80 effect. In other tests 6 mg neoantergan/kg decreased the effect 5-fold, and 2 mg decreased it 2.5-fold. In a few animals the neoantergan greatly diminished the intensity but not the area of blueing by 48/80. The relative inefficiency of circulating neoantergan in antagonizing 48/80 suggests that 48/80 may have a more direct action on capillary permeability; histamine activity was reduced 9-fold by 0.02 mg neoantergan/kg, whereas as much as 6 mg/kg was required for a 5-fold decrease of 48/80 activity. Moreover, doses up to 15 mg/kg, which is near the LD<sub>50</sub> of neoantergan, did not abolish the response to 48/80. It is possible that at the centre of the bleb, 48/80 itself is in a sufficiently high concentration to increase capillary permeability by direct action. Nevertheless, since the area of the 48/80 lesion is diminished by neoantergan, the drug in the concentrations obtaining at the edge of the lesion clearly act by liberating histamine. In the centre of the lesion, either the increase in permeability is not wholly due to histamine, or the available neoantergan is overwhelmed by histamine rapidly liberated by the high concentration of 48/80.

*Fixation of 48/80.* Superinjection of 0.2 ml. saline into lesions of various ages made by 2 µg of 48/80, failed to increase the lesion-diameter after 2-3 min. With larger doses, 48/80 remained free for a longer period, and superinjection increased the lesion-diameter after 10 min or more.

*Duration.* The increased permeability, as tested by late blueing, induced by a single dose, lasts for 7-10 min, after which it declines, so that after 20-25 min no blueing occurs. In the graph illustrating this recovery (Text-fig. 7*b*), the arrows indicate the period between 12 and 15 min, when the blueing ceases to be intense and becomes relatively feeble; the decrease in lesion-diameter does not fully indicate the recovery of the vessels, which is substantially complete at 12-15 min.

*Immunity.* The three experiments in Table 4 exemplify the immunity induced by 48/80 to test injection of the same substance. Thus in Expt. I, 2 µg partly, and 10 µg almost wholly, immunize to test doses of 10 µg. In Expt. II, 50 µg immunize to 50 µg to some extent after 30 min and well after 3 hr, whereas after 5 hr the immunity is wearing off. Immunity is maximum

between 1.5 and 3.0  
immunizes well to  
munity is at a maxi  
and is gone at 5-6

TABLE 4. Immunizing

Expt.	Immunizing and 48/80
I	48/80
II	Hist
III	Saline

*The site of action:*  
plexuses of blood  
general blueing w  
blueing along the  
the main reservoir  
with the smaller  
blood vessels ma  
body skin by the t  
from the skin of tl  
and a 10 mm les  
induced by enoug  
than that produc  
sumably liberate  
lesion (see Text-f  
dose of histamin  
vascular tissue, a  
48/80 must be cc  
Cross-immunit

ly animals respond poorly in the skin of histamine proportionally insensitive states of apparent insensitivity to the skin.

with 48/80 than with in before the injection of ns of 48/80 and a saline es being randomized in a sed in each group so that -fold decrease in the 48/80 ed the effect 5-fold, and organ greatly diminished re relative inefficiency of sts that 48/80 may have ine activity was reduced as 6 mg/kg was required s up to 15 mg/kg, which response to 48/80. It is a sufficiently high con- t action. Nevertheless, ntergan, the drug in the learly act by liberating rease in permeability is gan is overwhelmed by of 48/80.

into lesions of various diameter after 2-3 min. iod, and superinjection

ate blueing, induced by so that after 20-25 min- ery (Text-fig. 7b), the n the blueing ceases to in lesion-diameter does substantially complete

plify the immunity in- Thus in Expt. I, 2  $\mu$ g of 10  $\mu$ g. In Expt. II, and well after 3 hr, immunity is maximum

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between 1.5 and 3.5 hr (Expt. III). In the ear, a concentration of 20  $\mu$ g/ml. immunizes well to test doses of the same concentration. Here also, the immunity is at a maximum between 1.5 and 3.5 hr, and then passes off gradually and is gone at 5-6 hr.

TABLE 4. Immunizing action of intradermal 48/80 and histamine. Means of four to six lesions

Expt.	Immunizing agent and dose ( $\mu$ g)	Age of primary lesion (hr)	Mean diameter (mm) and intensity* of reaction to test dose of	
			48/80 (10 $\mu$ g)	Histamine (2 $\mu$ g)
I	48/80	0		
		2	8.5 + +	7.0 f.
		2	2.0 f.	2.0 f.
	10	2	8.0 f.	2.0 f.
		0.5	9 +	8.5 +
		3	1.5 f.	2.0 f.
		5	4 +	8.0 f.
	Histamine	5	9 + +	2 $\pm$
		3	9 + +	8 f.
II	Saline	5	8.5 + +	9 +
		0.5	10 + +	10 + +
		3	10.5 +	10.5 +
	48/80	5	10.5 +	10.5 +
		0.5	5.2 $\pm$	7.3 +
		1.5	1.8 +	2.0 f.
	Saline	2.5	0.0	0.7 $\pm$
		3.5	3.8 $\pm$	5.5 +
		4.5	3.3 +	6.5 +
III	48/80	5.5	7.2 +	6.7 +
		6.5	6.2 +	6.7 +
		2.5	8.7 + +	6.7 +
	Saline	2.5	8.7 + +	6.7 +
		0.5	5.2 $\pm$	7.3 +
		1.5	1.8 +	2.0 f.
	Saline	2.5	0.0	0.7 $\pm$
		3.5	3.8 $\pm$	5.5 +
		4.5	3.3 +	6.5 +

\* Intensity: symbols as Table 3.

*The site of action of 48/80.* The accumulation of blue at the level of the two plexuses of blood vessels in skin treated with 48/80, as compared with the more general blueing with histamine (Text-fig. 1) and the visible development of blueing along the larger vessels of similarly treated ears, suggests strongly that the main reservoirs of histamine available for liberation are closely associated with the smaller arteries and veins. The association of this histamine with blood vessels may also be inferred from the intensity of blueing induced in body skin by the two substances. The amount of histamine that can be extracted from the skin of the trunk is about 3  $\mu$ g/g (Feldberg & Miles, unpublished work); and a 10 mm lesion occupies about 150 mg skin. The intensity of blueing induced by enough 48/80 to give a lesion-diameter of 10 mm is far greater than that produced by injecting 0.5  $\mu$ g histamine (the amount that 48/80 presumably liberates) in about 0.35 ml. saline, a volume that will give a 10 mm lesion (see Text-fig. 3b). We conclude that after injection a great deal of this dose of histamine is ineffective because it is distributed through relatively non-vascular tissue, and that the endogenous histamine which can be liberated by 48/80 must be concentrated near or in the blood vessels.

*Cross-immunity with histamine.* As already noted, the lesions developing

after the injection of a substance into an already-injected site are variable and not easily measured. Cross-immunity is nevertheless obvious by reason of a decline in intensity or size or both, of the blue area. Tables 3 and 4 exemplify the results usually obtained. Histamine and 48/80 each induces a good immunity to itself; histamine induces only a fair immunity to 48/80, but 48/80 induces a good immunity to histamine. In the ear, concentrations of 20  $\mu\text{g}/\text{ml}$ . of both induced a good though not marked cross-immunity between 48/80 and histamine. It appears, therefore, that 48/80 immunizes both by liberating histamine, which in turn immunizes the susceptible vessels, and either by exhausting the histamine which can be liberated in the skin or by interfering with the mechanism of release of the bound histamine not liberated by the immunizing dose.

#### *Skin reactions to leukotaxine*

In most respects, the reaction of the blood vessels to leukotaxine were remarkably similar to those produced by 48/80. The chief difference lay in the very high concentration of leukotaxine required to induce thrombosis of the vessels.

*Intradermal injection in the trunk and the ear.* Like 48/80, leukotaxine induces in 3-5 min a round area of intense blueing, the diameter of which is in proportion to the log. dose in the constant-volume titration (Text-fig. 3d) and to the log. volume in the constant-amount titration (Text-fig. 5c). Leukotaxine appears to be strongly adsorbed to the skin tissues (Table 2); minimal blueing dose in 0.1 ml. is about 1  $\mu\text{g}$ ; inhibition of blueing at the centre of the bleb may occur with amounts from 10  $\mu\text{g}$  upwards, absent in some animals until 100-500  $\mu\text{g}$  is reached. This inhibition is not permanent; the area blues within 15-25 min. With large doses, 2 mg or more, small areas of permanent inhibition are produced. On cross-section, the blue leukotaxine blebs are like those of 48/80 (Text-fig. 1e).

In the ear, concentrations of 200  $\mu\text{g}/\text{ml}$ . and over induce a general dilatation of the small vessels within 30 sec, and a rapid blueing that starts first along the small vessels, then the larger veins and finally along the larger arteries within the bleb. When concentrations of 300  $\mu\text{g}$  or more per ml. are placed near one of the larger veins or arteries, a narrow band of vasoconstriction may appear within 1 min, disappearing after 1-2 min. With concentrations above 2.5 mg/ml., thrombosis of the vessels may occur at the centre of the bleb, though it is less severe and less regular in occurrence than that induced by 48/80. The blue areas are always approximately round (cf. Pl. 2B) whether made by intradermal or intralymphatic injection.

*Effect of anaesthesia and neoantergan.* Blueing is diminished in area and intensity during bromethol and urethane anaesthesia. Intravenous neoantergan, 3-8 mg/kg body weight, diminishes the leukotaxine effect from 1.5- to

3-fold (Text-fig. 5c of 48/80, but rather

*Time-course of the* 0.2 ml. saline into 16 min and most of with 10  $\mu\text{g}$  wholly of them had recovered

*Immunity.* Table 5. Three immunizations with leukotaxine were the lowest dose again.

TABLE 5. Immunization

Immunizing  
and dose  
Histamine

48/80

Leukotaxine

immunizing dose :  
immunity must be  
diameter of the  
moderately to hist  
and 48/80, and sl  
only moderately  
Leukotaxine imm

The study of incre  
pigs has shown th  
duces a gross incre  
next 5 min. In cc  
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ted site are variable and obvious by reason of a tables 3 and 4 exemplify each induces a good immunity to 48/80, but 48/80 concentrations of 20  $\mu\text{g}/\text{ml}$ . nity between 48/80 and zes both by liberating vessels, and either by e skin or by interfering e not liberated by the

s to leukotaxine were ef difference lay in the uce thrombosis of the

0, leukotaxine induces er of which is in pro- 1 (Text-fig. 3d) and to fig. 5c). Leukotaxine le 2); minimal blueing centre of the bleb may ne animals until 100- he area blues within permanent inhibition lebs are like those of

e a general dilatation hat starts first along g the larger arteries er ml. are placed near asoconstriction may oncentrations above : centre of the bleb, an that induced by (cf. Pl. 2B) whether

inished in area and travenous neoanter- : effect from 1.5- to

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3-fold (Text-fig. 5c); the degree of neutralization is of the same order as that of 48/80, but rather less marked.

*Time-course of the leukotaxine effect.* By the superinjection method, putting 0.2 ml. saline into 5  $\mu\text{g}$  lesions, all the leukotaxine appeared to be fixed in 16 min and most of it in the first 5-8 min. The capillaries of the skin treated with 10  $\mu\text{g}$  wholly regained their normal impermeability within 25 min; most of them had recovered after 10 min (Text-fig. 7a, b).

*Immunity.* Table 5 typifies the results of several tests of leukotaxine immunity. Three immunizing doses of each substance, histamine, 48/80 and leukotaxine were used, and immunity tested after 2 hr by superinjecting the lowest dose again. As is common in all such tests, immunity is best when the

TABLE 5. Immunity and cross-immunity induced in the skin by leukotaxine. Primary lesions 2 hr old. Means of four lesions

Immunizing agent and dose ( $\mu\text{g}$ )	Mean diameter (mm) and intensity* of reaction to test dose of		
	Histamine (2 $\mu\text{g}$ )	48/80 (5 $\mu\text{g}$ )	Leukotaxine (17 $\mu\text{g}$ )
Histamine	8	7.4 $\pm$	N.T.
	4	7.0 $\pm$	N.T.
	2	7.1 $\pm$	7.0 $\pm$
	0	10.0 $\pm$	7.4 $\pm$
48/80	20	3.4 $\pm$	2.0 $\pm$
	10	3.5 $\pm$	2.2 $\pm$
	5	8.0 $\pm$	4.6 $\pm$
	0	9.0 $\pm$	7.2 $\pm$
Leukotaxine	67	N.T.	N.T.
	33	N.T.	N.T.
	17	9.0 $\pm$	7.1 $\pm$
	0	9.5 $\pm$	7.8 $\pm$

\* Intensity: symbols as in Table 3.  
N.T. = No test.

immunizing dose is 4-10 times greater than the test dose. In this example immunity must be judged as much by decrease in intensity as by decrease in diameter of the blueing. Leukotaxine immunizes well to itself but only moderately to histamine and 48/80. Histamine immunizes moderately to itself and 48/80, and slightly to leukotaxine; whereas 48/80, though immunizing only moderately to histamine, immunizes well to itself and leukotaxine. Leukotaxine immunity is maximal in 1-2 hr, and has passed off by the 4th hr.

## DISCUSSION

The study of increased permeability in the skin of the trunk of blue guinea-pigs has shown that after a latent period of 1½-3 min, injected histamine induces a gross increase in capillary permeability, which is maximum within the next 5 min. In concentrations of from 1 to 100  $\mu\text{g}/\text{ml}$ . it can be characterized by the response of blue animal to varied doses in constant injection-volume

and to constant doses in varied injection-volume. By the superinjection of saline into histamine lesions of varying ages, and by the intravenous injection of dye at varying periods after the intradermal histamine, it can be shown that from 3 to 10 min free histamine is disappearing from the lesion, and the capillaries are recovering their normal low permeability. By the superinjection of histamine into recovered histamine-treated areas, it can be shown that as they recover, the vessels become partly immune to histamine; the immunity increases up to 2 hr, and declines after 4 hr. This immunity is not due to any interruption of the blood supply to the immunized tissues. With concentrations above 100  $\mu\text{g/ml}$ , no blueing due to increased permeability is detectable.

In the ear, the outstanding peculiarity is the high concentration required to produce even a 15 min inhibition of blueing at the centre of the lesion, and the transience of the effect with lower concentrations (p. 237 and Table 6).

TABLE 6. Comparison of the approximate minimal effective concentrations of histamine, 48/80 and leukotaxine in the skin of the trunk (10 mm blebs) and the ear (3 mm blebs)

Effect	Drug	Minimal effective concentration in $\mu\text{g/ml}$ .	
		Trunk	Ear
Blueing	Histamine	1-2	0.5
	48/80	2	5.0
	Leukotaxine	10	—
Temporary inhibition of blueing, and arterial constriction*	Histamine	10-30	50
	48/80	10-30	250
	Leukotaxine	100	300
Thrombosis of blood vessels	48/80	30-50	250
	Leukotaxine	2500	5000
Leak of free drug into surrounding lymphatic plexus	Histamine	—	1000
	48/80	—	>10000
	Leukotaxine	—	>10000

\* Presumed in trunk, demonstrable in ear.

In the skin of the trunk inhibition lasts for hours. For this relative permanence we postulated an arterial vasoconstriction, and therefore an absence of exudation, for periods longer than the duration of increased permeability. This relation does not hold in the ear because vasoconstriction is visibly relaxed within 3-7 min, and increased permeability lasts up to 8 min and may persist, though feebly, for 12-15 min. The relative insensitivity of the ear to this inhibitory effect may be a reflexion of its greater vascularity; either the injected histamine is swept away by the blood and lymph streams more quickly or much of the injected histamine is taken up by receptors in the numerous small vessels, so that on reaching the underlying larger vessels its concentration is too low for vasoconstriction. As Table 6 shows, the ear displays the same relative insensitivity towards 48/80 and leukotaxine, and presumably for the same reasons.

The action of 48/80 stances have a latent 'fixed' to the tissues permeability and are this time partly immune increases for 2 hr; a differences, which are fully for the action of blueing by histamine in the region of the a suggesting that both Secondly, whereas histamine the immunity of the vessels so that their susceptibility to circ has a direct action sensitive than injected as a potent histamine liberator Paton & Schachter, largely because insensitive to histamine-liberators complete extinction neutralization was histamine to the same and neoantigen-antibody neutralization, and to some 'pharmacological' than to a direct action from the ready available 'intrinsic' in Dale's acts. Our observations ever reason for this indicates that we are susceptible to inhibition of histamine.

Our observations of a histamine-liberator whose capacity to isolated leukotaxine capacity to increase 1939; Spector, 19

the superinjection of intravenous injection nine, it can be shown from the lesion, and the

By the superinjection can be shown that as amine; the immunity nity is not due to any sues. With concentra- eability is detectable. ncentration required ntre of the lesion, and (p. 237 and Table 6).

ons of histamine, 48/80 and ear (3 mm blebs)

imal effective ration in  $\mu\text{g/ml}$ .

k	Ear
	0.5
	5.0
	—
	50
	250
	300
	250
	5000
	1000
	>10000
	>10000

relative permanence on absence of exuda- permeability. This on is visibly relaxed ain and may persist, of the ear to this in- ; either the injected ore quickly or much nerous small vessels, entration is too low be same relative in- ably for the same

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The action of 48/80 in many ways resembles that of histamine. Both substances have a latent period of about 3 min. When blueing starts they become 'fixed' to the tissues; as blueing proceeds, the vessels recover their normal low permeability and are apparently normal at the end of 10–20 min. They are at this time partly immune to second doses of the same size, and the immunity increases for 2 hr; and after 4 hr it begins to decline. But there are marked differences, which raise the question how far the release of histamine accounts fully for the action of 48/80 on capillary permeability? First, the distribution of blueing by histamine is general, but that produced by 48/80 is concentrated in the region of the arteries and veins in both the skin of the trunk and the ear, suggesting that bound histamine is localized in or near these larger vessels. Secondly, whereas high concentrations of histamine inhibit blueing by altering the immunity of the vessels, high concentrations of 48/80 do so by damaging the vessels so that thrombosis occurs. Thirdly, the two substances differ in their susceptibility to circulating neoantergan; we have to assume either that 48/80 has a direct action on capillaries, or that the histamine it releases is less sensitive than injected histamine to neoantergan. Since 48/80 is well established as a potent histamine-liberator (Feldberg & Paton, 1951; Paton, 1951; Paton & Schachter, 1951) the second assumption is the more justified, particularly because insensitivity to antihistaminics is displayed by other known histamine-liberators (MacIntosh & Paton, 1949). Although in our experiments complete extinction of 48/80 blueing by neoantergan was impossible, partial neutralization was achieved by 200 times the dose required to neutralize histamine to the same extent. The parallelism of dosage-response in untreated and neoantergan-treated animals (Text-fig. 5b) is consistent with a true neutralization, and with the hypothesis that the unneutralized blueing is due to some 'pharmacological inaccessibility' of the liberated histamine rather than to a direct action of 48/80 on the capillaries. This inaccessibility may result from the ready adsorption of 48/80 to the tissues, and the concentration of the depots of available histamine near the blood vessels. This histamine is perhaps 'intrinsic' in Dale's (1948) sense that it is released from the cells on which it acts. Our observations are not exact enough to decide this point. But whatever reason for the relative inefficacy of neoantergan with 48/80, the fact indicates that we need not demand of a presumed histamine-liberator that its susceptibility to inhibition by neoantergan shall be of the same order as that of histamine.

Our observations on 48/80 provide a pattern of the intradermal behaviour of a histamine-liberator, for comparison with substances like leukotaxine, whose capacity to liberate histamine is in doubt. Menkin (1936, 1938a, b) isolated leukotaxine from sterile inflammatory exudates, and described its capacity to increase capillary permeability. Later work (Duthie & Chain, 1939; Spector, 1951) proved it to be a family of positively chemotactic

polypeptides, of which the larger members have this action on the capillaries. Rocha e Silva & Dragstedt (1941) postulated that leukotaxine acted via histamine, and Dekanski (1949) showed that on intradermal injection it increased the histamine equivalent in cat's skin, and that its 'blueing' action was neutralized by neoantergan. Menkin (1936, 1938a), and Cullumbine & Rydon (1946) deny the mediation of histamine because leukotaxine does not cause contraction in isolated smooth muscle, and the blueing is not antagonized by neoantergan (Cullumbine, 1947). We shall not, however, expect a histamine-liberator to cause isolated smooth muscle to contract. Menkin (1938a) showed that leukotaxine did not do so; but neither does 48/80 (Paton, 1951). Nor is Cullumbine's failure to detect a neoantergan effect by roughly quantitative tests unexpected. Leukotaxine in the skin can have a very shallow dosage-response slope, so that as much as a 10-fold drop in potency might diminish the diameter of the lesion by as little as 2 mm. Unless the neutralization test is made at several dose-levels in several animals, a genuine though small neutralization effect might well be missed. The relative insusceptibility of leukotaxine to neoantergan may well be determined by factors responsible for the similar behaviour of 48/80 and other liberators. Leukotaxine differs from histamine in spreading less readily through the tissues after local injection, as first recorded by Menkin, and in failing to induce inhibition at the centre of injection blebs, except in high concentrations. If it acts solely by histamine-liberation, inhibition of blueing may be impossible because there is not enough histamine to reach required concentration, even though it is localized round the vessels upon which it ultimately acts. An inhibiting dose of histamine, say 5  $\mu$ g, produces a lesion about 8 mm in diameter, involving about 60 mg of skin. Ignoring spread to adjacent tissues, we arrive at an average inhibiting concentration of 83  $\mu$ g histamine/g skin; whereas the normal content of extractable histamine in the skin of the trunk is of the order of 3  $\mu$ g/g (W. Feldberg & A. A. Miles, unpublished work). This histamine is presumably all available for liberation. Leukotaxine, except for its inability to induce thrombosis in moderate concentrations, behaves in most respects like 48/80. Both these substances resemble histamine in several respects, notably the similarity of the time-course of blueing (the lag period and duration of permeability) and in the induction and duration of immunity.

We can attach weight to these likenesses as a proof that leukotaxine acts through histamine if the effects are peculiar to histamine. This may well be so, but it should be noted that the time-course of blueing, and duration of permeability is shared by a large number of blueing substances besides 48/80, e.g. neoarsphenamine, stilbamidine, acetylcholine, peptone and hypotonic saline (unpublished work); and though some of these substances are probably histamine-liberators, the time course of their effect may reflect very general properties of vascular endothelium. The evidence obtained from the cross-

immunization test a similar reason, t is affected, and m cross-immunity b imply the same sit substance may w immunity betwee exhaustion of th liberated histamin

TABLE 7. 1

Ind  
His  
48/80  
Leu  
+

It should be no and 'immunizati tory state' becau in the skin by sul extended to cove may be the sam histamine wheali phenomena have of the blood sup circulating dye f But whereas in n several hours in taxine and 48/8 nervous origin d it lasts for sever a substance like

The cross-immn been due to dev the region of im of the three subs the modes of ac that the histai Although cross-i

tion on the capillaries. leukotaxine acted via dermal injection it in- at its 'blueing' action (a), and Cullumbine & e leukotaxine does not e blueing is not antag- not, however, expect e to contract. Menkin her does 48/80 (Paton, rgan effect by roughly skin can have a very 0-fold drop in potency as 2 mm. Unless the in several animals, a e missed. The relative ell be determined by and other liberators. lily through the tissues n failing to induce in- concentrations. If it ng may be impossible d concentration, even ultimately acts. An a about 8 mm in dia- o adjacent tissues, we ag histamine/g skin; the skin of the trunk ublished work). This otaxine, except for its ns, behaves in most histamine in several ueing (the lag period uration of immunity. hat leukotaxine acts This may well be so, ng, and duration of tances besides 48/80, tone and hypotonic stances are probably reflect very general ned from the cross-

immunization tests, which are summarized in Table 7, may be misleading for a similar reason, that in the endothelium there is only one kind of site which is affected, and made resistant, by a wide variety of substances. In addition, cross-immunity between substances other than histamine does not necessarily imply the same site of action. Cross-immunity between histamine and another substance may well be an expression of true histamine immunity; but cross-immunity between two presumed histamine-liberators may be due either to exhaustion of the available histamine, to the immunization of vessels by liberated histamine or to an inhibition of the mechanism of histamine release.

TABLE 7. Summary of tests of immunity and cross-immunity to increased capillary permeability in the skin

Induced by	Immunity		
	Tested with		
	Histamine	48/80	Leukotaxine
Histamine	++	+	+
48/80	+++	+++	+++
Leukotaxine	++	+	+++

+, ++, +++ = moderate, good and marked immunity.

It should be noted here that we have used the very general terms 'immunity' and 'immunization' in this connexion, rather than 'refractoriness' or 'refractory state' because we have applied them generally to drug-resistance induced in the skin by substances other than histamine. The terms can legitimately be extended to cover these phenomena; and though our histamine 'immunity' may be the same phenomenon as Lewis & Grant's (1924) refractoriness to histamine whealing in man, we are not in a position to equate them. The two phenomena have much in common; whealing was inhibited in man by occlusion of the blood supply for 10 min and our blueing is inhibited by withholding the circulating dye for 10 min. Both were relative, never demonstrably absolute. But whereas in man the refractory state lasts 5-10 min, the immune state lasts several hours in the guinea-pig. The immunity induced by histamine, leukotaxine and 48/80 in some respects resembles the immunity to whealing of nervous origin described by Grant, Pearson & Comeau (1935). In both cases it lasts for several hours, and both are explicable in terms of an exhaustion of a substance like histamine or H-substance increasing capillary permeability.

The cross-immunity we demonstrated was never very solid. This may have been due to deviations from the exact superposition of the test injection on the region of immunized tissue, and to the different degrees of tissue affinity of the three substances tested. It may, however, reflect a distinction between the modes of action; though as regards leukotaxine, it is probably significant that the histamine-liberator 48/80 immunized well against leukotaxine. Although cross-immunity should not be accepted as a sufficient single criterion

of similarity of action, in conjunction with other evidence it is strongly suggestive.

Our various tests of the increase of capillary permeability in the skin by the three substances, do not constitute a rigorous proof that the effect of 48/80 and leukotaxine on the capillary endothelium of the skin is mediated by histamine. Nevertheless, taking the evidence as a whole, our observations on these three substances are all consistent with the view that leukotaxine acts as a histamine-liberator in inflammatory lesions. The question whether histamine, through leukotaxine or some other endogenous liberator, is the sole mediator of the increased capillary permeability in inflammation, is less easy to answer. A given blood vessel made permeable by histamine or leukotaxine becomes substantially immune to the further action of the drugs within 20 min. The 'perpetuation of increased vessel permeability due to the gradual accumulation of peptides in the tissues' postulated by Spector (1951) in the natural course of inflammation must therefore be produced either by partial stimulation, and consequently only a partial immunization, of some parts of the vessel, leaving other parts for later stimulation; or, what is more consistent with the observed expansion of most progressive inflammatory lesions, by a gradual outward spread of leukotaxine to as yet unaffected vessels, leaving impermeable those already affected. But even with these refinements, the major role of leukotaxine is not necessarily established in inflammation, particularly infective inflammation. For example, the time-course of blueing by several clostridial exotoxins is quite different from that of leukotaxine (unpublished work); blueing may take an hour to develop and permeability persist for several hours; and cross-immunity with histamine and histamine-liberators is difficult to demonstrate. The behaviour of *Cl. oedematiens* exotoxin is singular, because the increased capillary permeability induced by a single dose persists for more than 30 hr. This observation probably has some bearing on the extensive oedema which accompanies infection by this microbe, but in this context it is chiefly interesting as indicating the existence of substances of pathological importance which appear to alter capillary permeability in a manner quite different from that of histamine or histamine-liberators.

#### SUMMARY

1. The increase in capillary permeability in the skin of the trunk, and ear of guinea-pigs was measured by the size and intensity of the blue lesion induced by the intradermal injection of histamine, the histamine-liberator 48/80, and leukotaxine, in animals with pontamine sky blue 6X in their circulation. In the trunk, the mean lesion-diameter from graded doses of these drugs in a constant volume is proportional to the log. dose; and for a constant dose in graded injection-volumes it is proportional to the log. volume.

2. The linear dose the basis of an assay dose' measurements three substances.

3. Histamine has high, affinity for skin injection fluid to the 48/80 and leukotaxine

4. All three substances injection, and their a 30 min. Between 15 begin to recover their doses of the drug. The

5. Histamine blue blueing is most in arteries and veins, located in these regions

6. High local concentration local vasoconstriction the time the constriction normal low permeability by thrombosing the l

7. Anaesthesia, should be secondary to a decrease of the capillaries to p

8. Circulating neo over 200 times less antagonism to both sub

9. The three substances The cross-immunity 48/80 and leukotaxine due either to histamine or inhibition

10. The similarity effect, and in the view that leukotaxine In all the tests made, paralleled by different bosing effect of strong these differences are i liberate histamine.

## 1. MILES

other evidence it is strongly

permeability in the skin by the proof that the effect of 48/80 of the skin is mediated by a whole, our observations on the view that leukotaxine acts on. The question whether endogenous liberator, is the sole in inflammation, is less easy by histamine or leukotaxine of the drugs within 20 min. due to the gradual accumulator (1951) in the natural ed either by partial stimulation, of some parts of the or, what is more consistent inflammatory lesions, by a unaffected vessels, leaving with these refinements, the established in inflammation, the time-course of blueing from that of leukotaxine develop and permeability in histamine and histamine-of *Cl. oedematiens* exotoxin ability induced by a single probably has some bearing on by this microbe, but in existence of substances of capillary permeability in a histamine-liberators.

in of the trunk, and ear of of the blue lesion induced mine-liberator 48/80, and in their circulation. In loses of these drugs in a and for a constant dose in 3. volume.

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2. The linear dosage-response to histamine, 48/80 and leukotaxine forms the basis of an assay method in the 'constant-volume' titration; 'constant-dose' measurements can be used to measure the affinity of skin tissue for the three substances.

3. Histamine has a relatively low, and 48/80 and leukotaxine a relatively high, affinity for skin tissue. Injected histamine spreads readily with the injection fluid to the subcutaneous tissues and lymphatic channels, whereas 48/80 and leukotaxine tend to remain in the skin.

4. All three substances increase capillary permeability within 3-5 min of injection, and their action is mostly finished in 10-15 min, and wholly so in 30 min. Between 15 and 30 min after the injection, the capillaries not only begin to recover their normal low permeability, but become immune to further doses of the drug. The immunity is greatest from 1-3 hr and declines after 4-5 hr.

5. Histamine blueing is general throughout the depth of the skin; 48/80 blueing is most intense in the region of arterioles, venules and the smaller arteries and veins, suggesting that the histamine available for liberation is located in these regions.

6. High local concentrations of histamine inhibit blueing by inducing severe local vasoconstriction during the period of increased permeability, so that by the time the constriction is relaxed the vessel walls have recovered their normal low permeability. High local concentrations of 48/80 inhibit blueing by thrombosing the blood vessels.

7. Anaesthesia, shock and chilling decrease blueing. The effect appears to be secondary to a decline in local intravascular pressures, and not to resistance of the capillaries to permeability-inducing substances.

8. Circulating neoantergan strongly antagonizes histamine blueing. It is over 200 times less effective with 48/80 and leukotaxine, but a definite antagonism to both substances is demonstrable.

9. The three substances induce a substantial cross-immunity to one another. The cross-immunity to histamine is presumably due to histamine liberated by 48/80 and leukotaxine. Cross-immunity between histamine-liberators may be due either to histamine immunization of the blood vessels, exhaustion of histamine or inhibition of the mechanism of histamine release.

10. The similarities in the time-course and duration of the permeability effect, and in the induction of cross-immunity, give strong support to the view that leukotaxine increases capillary permeability by liberating histamine. In all the tests made, the differences between leukotaxine and histamine were paralleled by differences between 48/80 and histamine, excepting the thrombosing effect of strong 48/80. Since 48/80 is an established histamine-liberator, these differences are not good evidence that substances displaying them do not liberate histamine.

We are greatly indebted to Dr E. J. de Beer at the Wellcome Research Laboratories, Tuckahoe, U.S.A., for a generous gift of the compound 48/80, and to our colleague Dr J. H. Humphrey for leukotaxine.

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EXPLANATION OF PLATES

PLATE 1

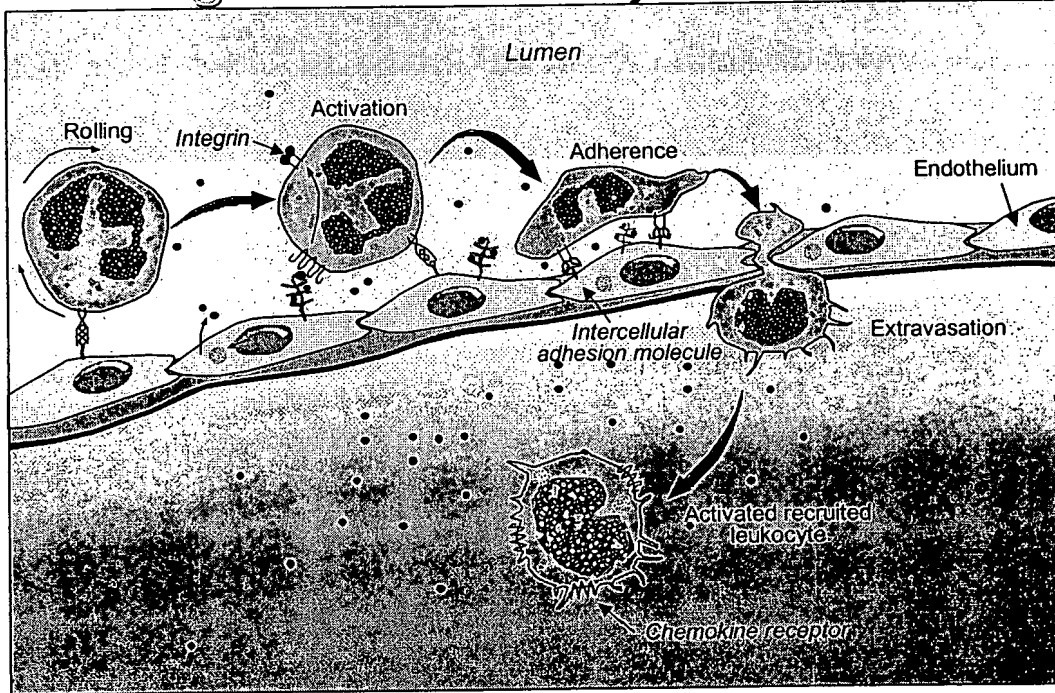
- A. Untreated ear of blued guinea-pig, showing the degree of initial opacity near the main vessels, due to the relative thickness of the ear in that region.  $\times 5$ .
- B. The same ear as in A after injection of strong histamine (1000  $\mu\text{g}/\text{ml}$ .) directly into the lymphatic plexus. The indian-ink stain on the skin at the top marks the injection site. The area of intense blueing is approximately wedge shaped, with the apex at the base of the ear.  $\times 5$ .

PLATE 2

- A. Ear of a blued guinea-pig injected with strong 48/80 (500  $\mu\text{g}/\text{ml}$ .). There is inhibition of blueing at the centre of the lesion, with the thrombosed superficial vessels in sharp focus.  $\times 5$ .
- B. Ear of a blued guinea-pig after injection of strong 48/80 (150  $\mu\text{g}/\text{ml}$ .) directly into the lymphatic plexus. The opacity round the main vessels on the left is due to the thickness of the ear. The area of intense blueing is approximately circular.  $\times 5$ .

## EXHIBIT B

### Regulation of Leukocyte Movement



## HUMAN CHEMOKINES: An Update

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**KEY WORDS:** structure, structure-activity relations, receptors, leukocyte migration, pathophysiology, HIV

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### ABSTRACT

Interleukin 8, the first chemokine to be characterized, was discovered nearly ten years ago. Today, more than 30 human chemokines are known. They are often upregulated in inflammation and act mainly on leukocytes inducing migration and release responses. The present review deals largely with the new developments of the last three years. Several structural studies have shown that most chemokines form dimers. The dimers, however, dissociate upon dilution, and the monomers constitute the biologically active form. Chemokine activities are mediated by seven-transmembrane-domain, G protein coupled receptors, five of which were discovered in the past three years. The primary receptor-binding domain of all chemokines is near the NH<sub>2</sub> terminus, and antagonists can be obtained by truncation or substitutions in this region. Major progress has been made in the understanding of chemokine actions on T lymphocytes that respond to several CC chemokines but also to IP10 and Mig, two CXC chemokines that selectively attract T cells via a novel receptor. Effects of chemokines on angiogenesis and tumor growth have been reported, but the data are still contradictory and the mechanisms unknown. Of considerable interest is the recent discovery that some chemokines function as HIV-suppressive factors by interacting with chemokine receptors which, together with CD4, were recognized as the binding sites for HIV-1.

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### INTRODUCTION

Chemokines constitute a large family of small cytokines with four conserved cysteines linked by disulfide bonds (Figure 1). Two subfamilies, CXC and CC chemokines, are distinguished according to the position of the first two cysteines, which are separated by one amino acid or are adjacent. Most chemokine sequences were derived from cDNAs encoding proteins of 92 to 125 amino acids with leader sequences of 20 to 25 amino acids. In humans, the genes of the CXC chemokines are clustered on chromosome 4, and those of the CC

**Table 1** Ligand selectivity of chemokine receptors

Receptors	New	Old nomenclature	Ligands <sup>a</sup>
CXC chemokines	CXCR1	IL-8R1 (type A)	IL-8
	CXCR2	IL-8R2 (type B)	IL-8, GRO $\alpha$ , $\beta$ , $\gamma$ , NAP-2, ENA78, GCP-2
	CXCR3	IP10/MigR	IP10, Mig
	CXCR4	LESTR, HUMSTR	SDF-1
CC chemokines	CCR1	RANTES/MIP-1 $\alpha$ R	RANTES, MIP-1 $\alpha$ , MCP-2, MCP-3
	CCR2a/b	MCP-1RA/B	MCP-1, MCP-2, MCP-3, MCP-4
	CCR3	EotaxinR, CC CKR3	eotaxin, RANTES, MCP-3, MCP-4
	CCR4	CC CKR4	RANTES, MIP-1 $\alpha$ , MCP-1
	CCR5	CC CKR5	RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$

<sup>a</sup>K<sub>d</sub> of 0.1 to 10 nM or Ca<sup>2+</sup> mobilization at <10 nM.

chemokines on chromosome 17. As indicated by the rapidly expanding literature, chemokines are increasingly studied because of their actions on leukocytes and their role in inflammation and immunity. Additional interest is arising from the recent discovery of a function of chemokines and chemokine receptors in HIV infection. Because of space limitations, we shall concentrate on new, biologically important findings on human chemokines reported during the past three years. For older reports, the reader may turn to our last, comprehensive review, which appeared at the beginning of 1994 and covered the literature up to the middle of 1993 (1). Several other reviews that have appeared since 1994 may also be consulted (2–4). The new, simplified nomenclature for chemokine receptors, which was elaborated at the 1996 Gordon Research Conference on “Chemotactic Cytokines” (Table 1), will be used.

## CHEMOKINE STRUCTURE

### *Three-Dimensional Structure of CXC and CC Chemokines*

The first chemokines for which the three-dimensional structure was determined are PF4 and IL-8. Their monomeric structures are very similar and comprise a NH<sub>2</sub>-terminal loop, three antiparallel  $\beta$ -strands connected by loops, and a COOH-terminal  $\alpha$ -helix. IL-8 forms globular dimers in solution consisting of a six stranded antiparallel  $\beta$ -sheet (made up of the three  $\beta$ -strands of each subunit) and two antiparallel helices lying across the  $\beta$ -sheet. The axis of symmetry is located between residues 26 and 26' at the center of strands  $\beta$ 1 and  $\beta$ 1' (5–7). PF4 forms an asymmetric tetramer by the dimerization of dimers of the IL-8 type (8). The structures of GRO $\alpha$  and NAP-2 are similar to that of IL-8, at both the monomer and dimer level (9–12). GRO $\alpha$  differs from IL-8 in the NH<sub>2</sub>-terminal region containing the ELR motif, the NH<sub>2</sub>-terminal loop extending between residues 12 and 23, and the turn between residues 31 and 36,

which is linked to the NH<sub>2</sub>-terminal region through the 9 to 35 disulfide bond. These regions are involved in receptor interaction (13), and the differences could determine receptor specificity.

The three-dimensional structure of MIP-1 $\beta$  (14) and RANTES (15, 16) consists of dimers formed by interaction of the NH<sub>2</sub>-terminal regions of the monomers yielding an elongated, cylindrical shape. The axis of symmetry is located between residues 10 and 10' in MIP-1 $\beta$  and 9 and 9' in RANTES. These residues are part of an additional, short antiparallel  $\beta$ -sheet formed by the strands  $\beta$ 0 and  $\beta$ 0'. The distribution of surface hydrophobicity differs markedly between CXC and CC chemokines (17), and this is believed to be the reason for the different mode of dimerization (7, 17). The core hydrophobicity clusters of CXC and CC chemokines, by contrast, are at equivalent positions, in agreement with the similarity of the three-dimensional structure of the monomers.

### *Monomers and Dimers*

Because most chemokines dimerize in solution, the dimer was generally assumed to be the biologically relevant form. Although the biological activities are observed at nanomolar concentrations, while the dissociation constants are mostly in the micromolar range (18, 19), this remained the prevailing view until proof was provided that IL-8 can function as a monomer (20). For this purpose an IL-8 analog was synthesized with N-methyl-Leu instead of Leu at position 25 to disrupt hydrogen bonding between the monomers. The methylated analog remains monomeric even at millimolar concentration and has nonetheless full activity on neutrophils (20). Its three-dimensional structure is similar to that of the subunits of the IL-8 dimer, indicating that the constraints imposed by dimer formation are not critical for the tertiary fold (21). Among the CC chemokines, monomeric forms of MIP-1 $\alpha$  were studied extensively and found to be biologically active (22–24). Data obtained by size exclusion HPLC, analytical ultracentrifugation, chemical cross-linking, and titration microcalorimetry support the conclusion that IL-8 and MCP-1, at physiological concentrations, occur predominantly as monomers (18, 25). Platelet basic protein (PBP) and its congeners including NAP-2 appear to behave in a similar manner (10). A different view was presented for MCP-1 based on the observations that chemically cross-linked dimers were active at nanomolar concentrations and that an antagonist obtained by NH<sub>2</sub>-terminal truncation formed a heterodimer with wild-type MCP-1 acting as a dominant negative inhibitor (26).

## NEW CHEMOKINES AND CHEMOKINE ACTIONS

Many new human chemokines have been discovered in the past few years, and considerable new information has been gathered about their activities on

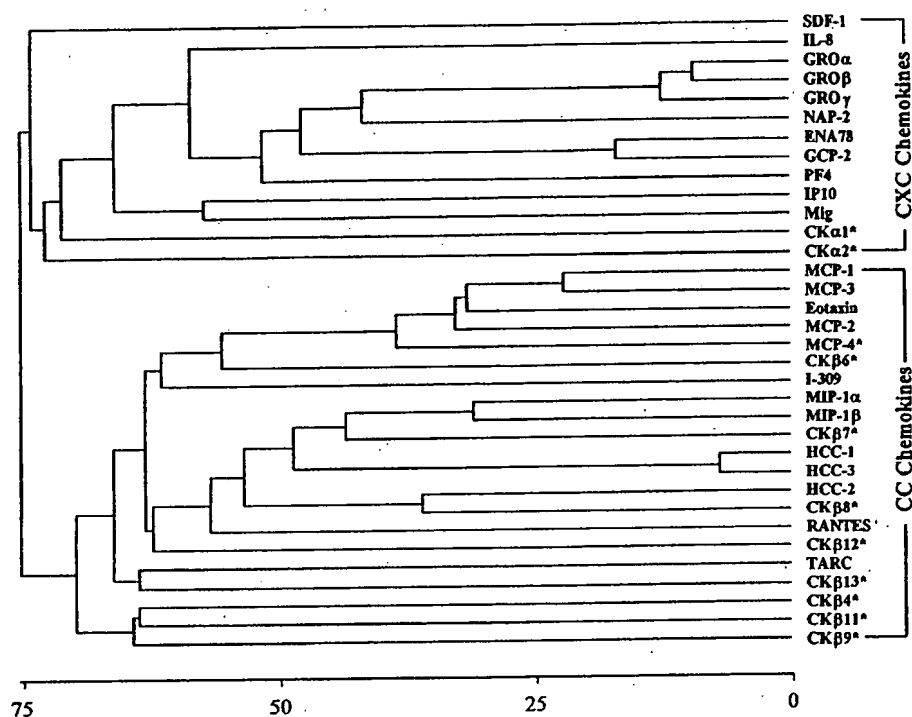
different types of leukocytes. Several of the more than 30 gene products, however, are known just by the cDNA-deduced amino acid sequence, and little information is available on biological effects. The dendrogram in Figure 1 presents all human chemokines described to date. Although the number has grown considerably, CXC and CC chemokines still fall into completely separate branches. Most new chemokines belong to the CC subfamily. They include: (i) eotaxin (27) and MCP-4 (28), which are structurally closely related to MCP-1; (ii) HCC-1 and a newly identified alternative splicing variant HCC-3, which are similar to MIP-1 $\alpha$  (29); (iii) TARC (thymus and activation-regulated chemokine), obtained from thymus-derived RNA, and reported to be chemotactic for T cell lines but not for blood T lymphocytes, monocytes, or neutrophils (30); and (iv) HCC-2, a CC chemokine with six instead of four cysteines located at identical positions as in the murine chemokines C10 (31), CCF18 (32), MRP-2 (33), and MIP-1 $\gamma$  (34). Several new chemokine cDNAs were isolated from human tissues within a large-scale cDNA sequencing program (Human Genome Sciences Ltd, Rockville, MD), and the mature proteins were expressed in insect cells (Figure 1). Some of the new CC chemokines are virtually inactive on granulocytes, monocytes, and lymphocytes, suggesting that they may stimulate precursor cells or other targets. It is important to realize, however, that the actual NH<sub>2</sub> terminus of these chemokines is unknown and that lack of activity may result from incorrect processing upon expression in insect cells.

The following subsections describe several newly discovered chemokines and some known ones for which interesting new properties were found. The chemokine receptors, to which reference is made, are described in a later section and Table 1.

### *The Monocyte Chemotactic Proteins*

MCP-1 was the first CC chemokine to be characterized biologically and shown to attract monocytes but not neutrophils (1). Two related proteins, MCP-2 (HC-14) and MCP-3, were subsequently identified (35), and MCP-4 was described only recently (28). The MCPs share a pyroglutamate proline NH<sub>2</sub>-terminal motif and are structurally closely related to each other and to eotaxin (56% to 71% amino acid sequence identity) (Figure 1). They have a broad spectrum of activity and attract monocytes (28, 36), T lymphocytes (37–39), and basophil leukocytes (1, 40–42). MCP-2, MCP-3, and MCP-4, in contrast to MCP-1, are also active on eosinophil leukocytes (28, 40, 42). These patterns can be explained with a minimum of three receptors: CCR1 recognizing RANTES, MCP-2, and MCP-3; CCR2 recognizing all MCPs; and CCR3 recognizing RANTES, MCP-3, MCP-4, and eotaxin. Monocytes and presumably also basophils express CCR1 and CCR2, and eosinophils express CCR1 and CCR3





**Figure 1** Structure similarity diagram of human CXC and CC chemokines. Similarity scores of the proteins were determined by the average linkage cluster analysis (181). The gap penalty, window size, filtering level, and K-tuple size parameters for pairwise alignments were set at 3, 10, 2.5 and 1, respectively. Distance to the branching points indicates the percent of sequence divergence. Highest pairwise similarity (7.5% divergence) is obtained for the CC chemokines HCC-1 and HCC-3, which differ only in the NH<sub>2</sub>-terminal region preceding the adjacent cysteines. Overall similarity between the two subfamilies of chemokines is 24.5% (75.5% divergence). Chemokines from Human Genome Sciences Ltd. are listed by their laboratory abbreviation, CK $\alpha$  or CK $\beta$  (for CXC and CC chemokines, respectively) followed by a number. GeneBank accession numbers for the sequences are (from top to bottom): U16752, M17017, J03561, M36820, M36821, M54995, L37036, P80162, M20901, X02530, X72755, X14768, X72308, U18941, P80075, M57502, X03754, J04130, Z49270, Z70293, Z70292, M21121, and D43767. The accession numbers for the chemokines marked with an asterisk are not available.

(2, 3, 43, 44). The picture may become more complex when CCR4 and CCR5 are considered, but the distribution of these receptors and their selectivity must first be studied in more detail. In basophils, MCP-1 is highly effective as a stimulus of histamine and peptido-leukotriene release, but it has only moderate chemotactic activity, whereas RANTES is a strong chemoattractant and a weak inducer of mediator release. This suggests that CCR1 and CCR2 are functionally different, and indeed, maximum migration and release are obtained with MCP-3 that binds to both receptors (2).

Of particular interest are the effects of the MCPs on lymphocytes. Studies on human CD4<sup>+</sup> or CD8<sup>+</sup> T cell clones (28, 37) and human blood lymphocytes (38, 39) show that all four MCPs are potent attractants for activated T lymphocytes. Under similar conditions, MCP-1, MCP-3, and MCP-4 attract more cells than do RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  across bare or endothelial cell-coated filters. All MCPs also induce a transient *B. pertussis* toxin-sensitive rise in the cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (28, 37). Conditioning with IL-2 markedly enhances the expression of CCR1 and CCR2 and the chemotactic responsiveness to RANTES and MCP-1 (45) (see *Lymphocyte Recruitment*). Similar migration responses are observed for natural killer (NK) cells (46) and dendritic cells (47), and the MCPs were also found to induce [Ca<sup>2+</sup>]<sub>i</sub> changes and exocytosis of granzyme A and N-acetyl- $\beta$ -glucosaminidase in cloned human NK cells (48). In addition, several CC chemokines including the MCPs were reported to enhance target cell lysis by blood-derived NK cells (49).

### *Eotaxin*

A CC chemokine acting on eosinophils and termed *eotaxin* was originally isolated from the bronchoalveolar fluid of allergic guinea-pigs (50). Murine (51) and human homologs (27) were subsequently cloned. They share over 60% sequence identity with guinea-pig eotaxin and are equally selective for eosinophils. Human eosinophils express high numbers of a receptor for eotaxin, CCR3, which was cloned by three independent groups (43, 44, 52). Binding studies have shown that eotaxin as well as RANTES and MCP-3 recognize this receptor (43), in agreement with their ability to attract CCR3-transfected cells (44). In addition, cross-desensitization experiments with eosinophils suggest that CCR3 recognizes MCP-4 as well (27, 28). MCP-4 is very active on eosinophils and is equivalent to eotaxin as chemoattractant and superior to MCP-3 in desensitizing eosinophils toward eotaxin (28). In contrast to other CC chemokines, eotaxin has a high degree of selectivity for its receptor. It is inactive on neutrophils and monocytes, which do not appear to express CCR3 (44, 52, 53) but has weak-to-moderate chemotactic activity toward IL-2-conditioned T lymphocytes (28). Eotaxin exclusively attracts eosinophils when

applied in vivo (27), and its expression is enhanced in animal models of allergic inflammation (50, 54) and in tissue cells at sites of eosinophil accumulation in humans (27). Northern blot analysis showed constitutive expression of eotaxin in human small intestine, colon, heart, kidney, and pancreas; major amounts of this chemokine are believed to be produced by epithelial and endothelial cells as well as eosinophil leukocytes (53). Due to its preferential, powerful action on eosinophils and its occurrence in different species, eotaxin is considered a most relevant chemokine in the pathophysiology of allergic conditions and asthma (55).

### *IP10 and Mig*

IP10 is a CXC chemokine that was identified several years ago as the product of a gene induced by interferon- $\gamma$  (IFN $\gamma$ ), which was found to be expressed in delayed-type hypersensitivity reactions of the skin (56, 57). For a long time the biological activity of this chemokine remained unclear. Another IFN $\gamma$ -induced human CXC chemokine, Mig, was later described (58). IP10 was reported to attract human monocytes, T lymphocytes, and NK cells (49, 59), and Mig was shown to attract tumor-infiltrating T lymphocytes (60). A receptor that is specific for IP10 and Mig, CXCR3, was recently cloned (see *CXC Chemokine Receptors*) and found to be selectively expressed on activated T lymphocytes that appear to be the only target cell for the two IFN $\gamma$ -inducible chemokines (61). The restricted expression and the selectivity for a single receptor on T cells suggest that IP10 and Mig are involved in the regulation of lymphocyte recruitment and the formation of the lymphoid infiltrates observed in autoimmune inflammatory lesions, delayed-type hypersensitivity, some viral infections, and certain tumors.

### *SDF-1*

The CXC chemokine SDF-1 (stromal cell-derived factor 1) occurs in two alternative splicing variants, SDF-1 $\alpha$  and SDF-1 $\beta$ , that were cloned from mouse bone marrow stromal cell lines (62, 63). SDF-1 $\alpha$  stimulates the proliferation of B cell progenitors and was also termed PBSF (pre-B cell growth stimulating factor) (63). Murine SDF-1 $\alpha$  was purified as a lymphocyte chemoattractant from a stromal cell culture supernatant (64). A homologous gene of human origin coding for both SDF forms was later characterized, and SDF-1 $\alpha$  was shown to be the more abundant variant (64a). Mature human and murine SDF-1 $\alpha$  consist of 68 amino acids and differ only at position 18 (valine in the human and isoleucine in the murine protein). Subsequent studies showed that synthetic human SDF-1 stimulates monocytes, neutrophils, and peripheral blood lymphocytes, as is indicated by  $[Ca^{2+}]_i$  changes and chemotaxis (64, 65). SDF-1 binds to CXCR4, a former orphan receptor cloned in several laboratories (66–70), (see

*CXC Chemokine Receptors*), and induces  $\text{Ca}^{2+}$  mobilization in CHO cells that stably express this receptor (65, 71). No cross-desensitization is observed with other chemokines, which underlines the selectivity of CXCR4. In transfected cell lines coexpressing CXCR4 and CD4 and in blood lymphocytes, SDF-1 is a powerful HIV-suppressive factor (see *HIV Replication*). Mice lacking the SDF-1 gene die perinatally and present multiple defects of development, including a severe reduction of B cell and myeloid progenitors in the bone marrow, in addition to a septal defect in the heart. These findings suggest that SDF-1 may display additional functions that are not typical for chemokines (72).

## CHEMOKINE RECEPTORS

Chemokines act via seven-transmembrane-domain (7TM) receptors (1, 3), which form a distinct group of structurally related proteins within the superfamily of receptors that signal through heterotrimeric GTP-binding proteins (Table 1). More than by the overall sequence identity, relatedness is manifested by a number of conserved structural motifs mainly found within the transmembrane domains. The most important of these motifs are: TD(X)YLLNLA (X2)DLLF(X2)TLP(X)W in TM 2, the  $\text{NH}_2$ - and COOH-terminal extensions of the DRYLAIVHA-motif in TM 3 and the second intracellular loop, PLL(X)M(X2)CY in TM 5, W(X)PYN in TM 6, and HCC(X)NP(X)IYAF(X)G(X2)FR in TM 7. In addition, all chemokine receptors have two conserved cysteines, one in the  $\text{NH}_2$ -terminal domain and the other in the third extracellular loop (Cys<sup>30</sup> and Cys<sup>277</sup> in CXCR1) that are assumed to form a disulfide bond critical for the conformation of the ligand-binding pocket. On the basis of the overall sequence identity, two subgroups can be recognized: CXC chemokine receptors with 36–77% and CC chemokine receptors with 46–89% identical amino acids (Figures 2 and 3).

### *CXC Chemokine Receptors*

Two receptors for IL-8, CXCR1 (IL-8RA/R1) and CXCR2 (IL-8RB/R2), are expressed on neutrophils. They share 77% identical amino acids, and their genes are colocalized on chromosome 2q35 (73, 74). One receptor, CXCR2, has high affinity for IL-8 and all other CXC chemokines that attract neutrophils (e.g. the GRO proteins, NAP-2, etc.), while the other, CXCR1, has high affinity for IL-8 only (1). IL-8 receptors are also found on monocytes, basophils, and eosinophils, but the responses of these cells to IL-8 are much weaker than those of neutrophils (1). In T lymphocytes, expression of both IL-8 receptors is revealed by RT-PCR but not by Northern blotting (75, 76), suggesting that the numbers are low. Using monoclonal antibodies and FACS analysis, it was observed that CXCR1 and CXCR2 are present on all neutrophils and monocytes,

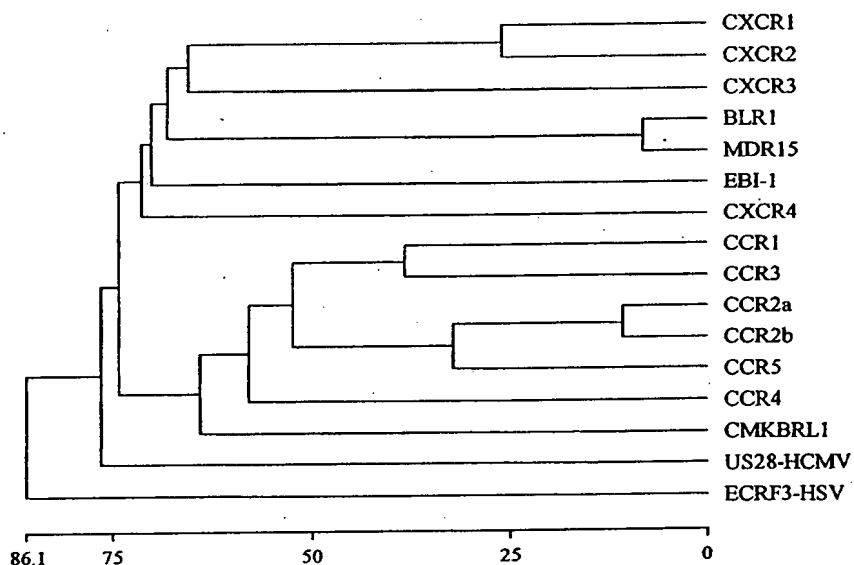


Figure 2 Structure similarity diagram of human chemokine receptors. Similarity scores were determined as described for Figure 1. The highest degree of divergence (86.1%) with respect to all the other receptors is observed for herpesvirus saimiri ECRF3 and the highest pairwise similarity (8.3% divergence) for BLR1 and MDR15. GeneBank accession numbers for the sequences are (from top to bottom): M68932, M73969, X95876, X68149, X68829, L08176, X71635, L10918, U51241, U03882, U03905, X91492, X85740, U28934, L20501, and S76368.

but only on a minority of lymphocytes. They are found in low numbers on NK and CD8<sup>+</sup> T cells, with high donor-to-donor variation, but they are absent in CD4<sup>+</sup> T cells or B cells (77, 78). Interestingly, while both receptors are present in similar numbers on neutrophils, CXCR2 appears to prevail on other leukocytes (78). Analysis of transendothelial migration shows that MCP-1 attracts CD26<sup>+</sup> T cells while the cells responding to IL-8 are CD26-negative (77). Expression of CXCR1 and CXCR2, as assessed by immunocytochemistry or RT-PCR, was also reported in cultured melanocytes and fibroblasts (75), epithelial cells in inflamed skin (79), and fibroblasts and smooth muscle cells of burn lesions (80). There is no evidence for a functional role of the expressed receptors, however.

Chimerae between the rabbit CXCR1 and the human CXCR2 were used to show that the NH<sub>2</sub>-terminal domain determines, to a large extent, ligand selectivity (81, 82). Receptors carrying the NH<sub>2</sub>-terminal domain of CXCR1 are selective for IL-8, whereas those carrying the NH<sub>2</sub>-terminal domain of CXCR2 have high affinity for other CX<sub>C</sub> chemokines as well. Alanine scanning

TM1  
MSNITDPOWMDFDLNFATGMPADEDYSPCKLE-TETLNKYVVIATLALVLLSLLGSLNHLVLYSRVGRSMVTVYLNALAD 85  
MEDFNRESDFEDPKWGEDLNYSYSSSTLPPFLDAAPOEPE-SLEINKYFVVIWVAVVLLSLLGSLNHLVLYSRVGRSMVTVYLNALAD 94  
MYLEVSDHQVLDNAEVAALLENFSSSYDGENSDYDSCCTPPCKODFSLNFRAPALPSTLHLLGULGNANVLLSRRRLSTSTTFULHVAAD 99  
85  
MGIISIVTSDNYTEEMSGDYSMEPECKREENANFNIPLFTIPSTLHLLGULGNANVLLSRRRLSTSTTFULHVAAD 85  
METPNTEDYDTEFDYG-----DATPKCNVTRAFQAQLLPLPSVAVVILVAVNVLVAVVQYKRLKNMTEJLYLNALISD 80  
MTSLDVTETFGTTSYDD-----VGLLCKADTRALMAQVFPPLPSVAVVILVAVNVLVAVVQYKRLKNMTEJLYLNALISD 80  
MLSTSRBRFIINTNESGHEVTTFDDYD-----YGAPCKFDVKYQIAQQLPLPSVAVVILVAVNVLVAVVQYKRLKNMTEJLYLNALISD 88  
MDYQVSSPIYDINYY-----TSEPCKINVKQIAARLLPLPSVAVVILVAVNVLVAVVQYKRLKNMTEJLYLNALISD 77  
MNPDTIADTTLDESISYNYLYYES-----IPKPCCKEGIKAFGELFLPELMSVAVVILVAVNVLVAVVQYKRLKNMTEJLYLNALISD 85

TM2  
TM3  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 183  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 192  
TELVITLPIAAV-DAVQVVRGSGFLCKVAGALFNFNFAGALLACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 198  
LLPVITLPIAAV-DAVQVVRGSGFLCKVAGALFNFNFAGALLACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 184  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 178  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 178  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 185  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 175  
LEPALTUHTHAA-SKVNGMHKGTFCKQVSVLLKEVNFSGIILFACISVDVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 182

TM4  
TM5  
SPV-C---YEVLGNDTAKRMVLRILPHTPFIYELFVNLFPCKGFTLRTPFAHMGQKH-RAMRVIFAVVLIPLILCPLVNLVLLATILMRTQVIOETCE 278  
SPA-C---YEDMGNTANWMLLRILPQSPFIYELFVNLFPCKGFTLRTPFAHMGQKH-RAMRVIFAVVLIPLILCPLVNLVLLATILMRTQVIOETCE 287  
NATHCOYPPQVG-----RTALRVLOLVAPLALLVLMVAYCAVHILAVLVRGQRLL-RAMRVIVVVVAVVAFALCPVHLVVVLIHMDLALARNCG 292  
YI--CDAPY-----NDLVVVVQFQHIMVLIILBIVILSCILISKSHSKGQKH-RAMRVITVILILAFACPLPKIGISILFILLEIIOGCE 276  
THEICSLHPPH--ESLREKFLQALKLPLGLVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 274  
EETLCSALYPE--DVTYSRHPHTLRMTIPCLVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 274  
SVVCGPYFPR-----CANFHTIMENILGLVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 278  
LHVTCSHFFPY--SQQPMKNFQTLKIVILGLVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 273  
NHTYCKTKYSL--NST-LKAVLSSLEINILGLVLAIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 277

TM6  
TM7  
RNNIGRALDAEILGLPLSCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 350  
RRNHIDRALDAEILGLPLSCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 360  
RESRVDVAKSVSGLYMCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 368  
FENTVHWISIEALAFPCCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 352  
QSRHDLAVQVREVIATPCCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 355  
RSKHLDLVLYVREVIATPCCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 355  
STSLDQATOVREVIATPCCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 360  
SSNLDQAMONREVIATPCCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 352  
FERYLDVAIQAREVIATPCCLNHLIVHATRTLTOKRHL-VKPVCLGCGHLSMNLSPFLFRQAYHPNNS 360

was cloned from a cDNA library derived from an Epstein-Barr virus-infected Burkitt's lymphoma, and shown to be expressed exclusively in B and T cell lines (103). Another receptor, CMKBRL1 (chemokine beta receptor-like 1) shows interesting similarities with chemokine receptors. Its gene is located on chromosome 3p21 together with other CC chemokine receptors, and transcripts are found in leukocytes as well as in lymphoid and neural tissues (104, 105).

Some chemokine receptors may mediate functions that are unrelated to cell migration, as is exemplified by two receptors of viral origin. US28 consists of 354 amino acids and is encoded by human cytomegalovirus. It recognizes several CC chemokines, including MCP-1, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  (87, 106). Conversely, ECRF3, a 321 amino acid protein encoded by herpesvirus saimiri, recognizes IL-8, GRO $\alpha$ , and NAP-2, but not CC chemokines (107). It is interesting that these viral receptors discriminate between CXC and CC chemokines although they share less than 30% identical amino acids with the human chemokine receptors. In human fibroblasts, cytomegalovirus induces the expression of IL-8 receptors and shows enhanced replication in receptor-expressing cells that are exposed to IL-8 (108). This suggests that the expression of chemokine receptors in infected cells may be of advantage for the replication of some viruses (3, 108).

### *Receptor Function and Signal Transduction*

Our review of this topic is largely restricted to neutrophils and cell lines transfected with the IL-8 receptors, which were studied more extensively. Signaling by chemokine receptors depends on coupling to *Bordetella pertussis* toxin-sensitive G-proteins. Experiments with COS-7 cells in which IL-8 receptors were cotransfected with different G-proteins have shown that CXCR1 and CXCR2 couple to G $\alpha_{12}$ , G $\alpha_{13}$ , G $\alpha_{14}$ , G $\alpha_{15}$ , and G $\alpha_{16}$  but not to G $\alpha_q$  or G $\alpha_{11}$  (109). Studies on CC chemokine receptors are less advanced. CCR1 was shown to couple to G $\alpha_{14}$  but not G $\alpha_{16}$ , while CCR2b couples to both G-proteins. No coupling to these G-proteins was observed for CCR2a, however, suggesting differences in G-protein usage (90).

The function of CXCR1 and CXCR2 was studied in Jurkat cells stably transfected with one or the other cDNA. CXCR1-expressing cells bind IL-8 with high affinity, and GRO $\alpha$  and NAP-2 with low affinity, while CXCR2-expressing cells have high affinity for all three ligands. Both types of transfectants respond equally well to IL-8, as shown by [Ca<sup>2+</sup>]<sub>i</sub> changes and chemotaxis, and no difference is observed in the function of CXCR2 after stimulation with IL-8, GRO $\alpha$ , or NAP-2. CXCR1-transfected cells migrate in response to GRO $\alpha$  and NAP-2, which bind with low affinity, provided that high concentrations are used (110). Similar experiments show that both receptors activate the p42/p44 MAP kinase (111). These results indicate that CXCR1 and CXCR2 signal and

function independently of each other. Monoclonal antibodies that selectively block CXCR1 or CXCR2 were used to study the function of the individual receptors in neutrophils. Both IL-8 receptors trigger  $[Ca^{2+}]_i$  changes, chemotaxis and granule exocytosis, whereas phospholipase D activation and the respiratory burst are only observed after stimulation of CXCR1 (112). These observations are in agreement with a study showing that in human neutrophils phospholipase D is activated by stimulation with IL-8, but not with GRO $\alpha$  or NAP-2 (113). All three chemokines, on the other hand, induce similar  $[Ca^{2+}]_i$  changes and patterns of phosphorylation. Activation of p42/44 MAP kinase is also observed in cells transfected with CCR2 cDNA after stimulation with MCP-1 (114).

There is strong evidence for a role of phosphatidylinositol 3-kinase (PI3K) in chemokine-mediated signal transduction (115, 116). PI3K isoforms may become activated directly by interaction with the G-protein  $\beta\gamma$  subunit or by small GTPases, Src-related tyrosine kinases, or phosphotyrosines that bind to the SH2 domain of PI3K. Phosphatidylinositol lipids phosphorylated at the 3-OH position are supposed to trigger a variety of cellular responses (117). A study on murine pre-B cells transfected with CXCR1 showed that the small GTPase Rho is implicated in IL-8-mediated adhesion to fibrinogen (118), suggesting that IL-8 receptors can activate small GTPases, which in turn regulate cytoskeletal rearrangement, phospholipase D activation, and induction of the respiratory burst (117). Leukocyte responses to chemokines are characteristically transient, and the receptors become rapidly desensitized. Phosphorylation of serines and threonines in the COOH-terminal region of CXCR1 and CXCR2 correlates with homologous desensitization after stimulation with IL-8 or GRO $\alpha$ , respectively (1, 119, 120). Rat basophilic leukemia cells (RBL-2H3) cotransfected with CXCR1 and the C5a receptor were used to show that heterologous desensitization correlates with COOH-terminal phosphorylation of the receptors (121).

## STRUCTURE-ACTIVITY RELATIONS

### *CXC Chemokines*

The short sequence Glu-Leu-Arg (ELR motif), which precedes the first cysteine in all CXC chemokines that act on neutrophils is essential for binding and activation of both IL-8 receptors (CXCR1 and CXCR2). Additional structural domains, however, are required because short peptides containing the ELR motif are inactive, and neither IP10 nor MCP-1 can be converted into neutrophil-activating chemokines by introduction of the ELR motif (1). Active CXC chemokines have a short NH<sub>2</sub>-terminal domain, and it has been suggested that if the sequence is extended, it can fold over the ELR motif and prevent its recognition by the receptor (10). Studies with IL-8 have shown that the arginine



adjacent to the first cysteine is very sensitive to substitution (1). It is interesting to note that three CXC chemokines, IP10, Mig, and SDF-1, that were recently shown to act via novel CXC chemokine receptors (61, 65) have a conserved arginine before the first cysteine. The Arg-Cys-X-Cys motif may be a general requirement for the binding to CXC chemokine receptors.

Several attempts have been made to define the structural elements for high-affinity binding to the IL-8 receptors. Studies with synthetic IL-8 analogs with single or double amino acid substitutions and hybrids between IL-8 and the inactive IP 10 have been performed to establish the minimal requirements for activity (13, 122). In addition to the disulfide bridges and the ELR motif, the NH<sub>2</sub>-terminal loop region (residues 10–17) and the Gly<sup>31</sup>-Pro<sup>32</sup> motif in the  $\beta$ -turn containing the third cysteine (residues 30–35) were found to be of primary importance (13, 19). Single residue mutations and chimeric proteins between IL-8 and CXC chemokines with low affinity for CXCR1, were used to identify the structural determinants for recognition of CXCR1 and CXCR2 (123–127). Of particular interest is the NH<sub>2</sub>-terminal loop (residues 10–17 in IL-8), since structural analysis reveals significant differences in this region between monomeric IL-8 and monomeric GRO $\alpha$  or NAP-2 (9, 11, 12). Mutations of human and rabbit IL-8 highlight the importance of Tyr<sup>13</sup> and Lys<sup>15</sup> for high affinity binding to CXCR1 (123). Mutants of IL-8 and GRO $\alpha$  with reversed receptor selectivity were obtained by exchanging the NH<sub>2</sub>-terminal loops of the two chemokines (residues 10–17 of IL-8 and 12–18 of GRO $\alpha$ ) and residues preceding the fourth cysteine (Glu<sup>48</sup> and Leu<sup>49</sup> of IL-8, and Ala<sup>50</sup> of GRO $\alpha$ ) (127).

The substitution of Tyr<sup>28</sup> and Arg<sup>30</sup> in MCP-1 by the corresponding residues in IL-8, Leu and Val, was reported to lower the activity toward monocytes and to confer neutrophil chemotactic activity to the CC chemokine (128). Conversely, replacement of Leu<sup>25</sup> and Val<sup>27</sup> in IL-8 by tyrosines, the corresponding residues of RANTES (129), or substitution of Leu<sup>25</sup> by a modified cysteine were reported to yield mutants with CC chemokine activity (130). Using synthetic mutants, we were unable to confirm the results obtained by substitutions with natural amino acids (128, 129), and we found that the weak activity that IL-8 normally has on monocytes (131) was not affected by the substitutions (I Clark-Lewis, B Dewald, unpublished observation).

### *CC Chemokines*

The NH<sub>2</sub>-terminal region of MCP-1 is of critical importance for receptor recognition and activation. The situation is similar to that of IL-8 and its analogs that activate neutrophils, but the structural requirements are more strict because the entire sequence of 10 residues preceding the first cysteine is required for full activity (19, 132). Truncation or elongation of the NH<sub>2</sub>-terminal sequence leads to considerable loss of activity, but the NH<sub>2</sub>-terminal pyroglutamate can

be replaced by several other noncyclic amino acids. This is particularly interesting because MCP-2, MCP-3, and MCP-4 share with MCP-1 the NH<sub>2</sub>-terminal pyroglutamate and high affinity for CCR2.

The role of the NH<sub>2</sub>-terminal domain for MCP-1 activity was studied with a series of NH<sub>2</sub>-terminally truncated analogs, MCP-1(2-76) to MCP-1(10-76), of the full-length form of 76 residues. Deletion of the NH<sub>2</sub>-terminal pyroglutamate, yielding MCP-1(2-76), results in a marked, at least 50-fold decrease in activity on monocytes (132) and basophils (133), and deletion of the next residue leads to total loss of activity. Analogs with deletions of 3 or 4 residues, MCP-1(4-76) and MCP-1(5-76), are active again on both cells, while all further truncation analogs, MCP-1(6-76) through MCP-1(10-76), are inactive. A surprising observation was that MCP-1(2-76), the analog without NH<sub>2</sub>-terminal pyroglutamate, is a powerful stimulus for eosinophil leukocytes, which do not express CCR2 and do not respond to full-length MCP-1 (133). On further truncation, the activity on eosinophils changes in the same way as in monocytes and basophils. It can be assumed that the effects on eosinophils are mediated via CCR3, and it is remarkable that MCP-1 acquires activity on these cells only after NH<sub>2</sub>-terminal deletion, whereas MCP-2, MCP-3, and MCP-4, which share the NH<sub>2</sub>-terminus with MCP-1, are potent attractants in their full-length form.

Several of the truncated MCP-1 analogs, MCP-1(9-76) and MCP-1(10-76) in particular, act as antagonists presumably by blocking CCR2, and they prevent the responses to MCP-1, MCP-2, and MCP-3, but not to RANTES, MIP-1 $\alpha$ , or MIP-1 $\beta$  (132). Analogous studies performed with RANTES and MCP-3 yielded antagonists for multiple CC chemokine receptors (95). RANTES(9-68) and MCP-3(10-76) inhibit receptor binding and functional activities of MCP-1, MCP-3, and RANTES. The decreased selectivity of the truncated analogs, RANTES in particular, suggests that receptor specificity is dictated by residues within the NH<sub>2</sub>-terminal domain, which are lost upon truncation, while other structural determinants assure the interaction with multiple receptors. Two additional CC chemokine antagonists were reportedly obtained by NH<sub>2</sub>-terminal elongation: MCP-3 with three additional residues, Arg-Glu-Phe, which blocks the activity of MCP-3 (134), and RANTES with an additional methionine, which blocks the activity of RANTES and MIP-1 $\alpha$ , but not of MCP-1 or IL-8 (135).

These observations demonstrate the critical role of the sequence preceding the first cysteine for the binding and function of MCP-1, MCP-3, and RANTES, and, together with former evidence on CXC chemokines (1), emphasize the overall importance of the NH<sub>2</sub>-terminal domain for the biological activity of all chemokines. Interesting differences are nevertheless apparent between CXC and CC chemokines. Minimal modifications of the NH<sub>2</sub> terminus can drastically reduce or even qualitatively change the activity of CC chemokines, while

truncation up to the ELR motif progressively enhances the potency of IL-8 and other CXC chemokines (1). In both cases, elimination of most residues in the short NH<sub>2</sub>-terminal stretch or modification of recognition motifs yields derivatives that still recognize the receptor but do not induce functional responses and thus act as antagonists.

## PERSPECTIVES

We conclude by highlighting some new developments and concepts of potential interest. For areas where progress has been slow and areas that were reviewed recently, only a few indicative references will be given. Research on chemokines has provided considerable insight into the mechanism of diapedesis, and the ability of endothelial cells to generate attractant chemokines has been recognized as a fundamental process. Outstanding reviews have been published on this subject (136–138). Of interest is the potential activity of chemokines on myeloid progenitor cells. MIP-1 $\alpha$  was described early on as a regulator of hematopoiesis (139) and inhibitor of stem cell proliferation (140). Although the disruption of the MIP-1 $\alpha$  gene in mice does not appear to cause cellular abnormalities in the bone marrow or blood (141), chemokines are still considered as potential stimuli of leukocyte production and release from the bone marrow. Another major subject is the role of chemokines in tumors. It is well documented that transformed cell lines produce high amounts of different chemokines for which anti-tumor as well as tumor-promoting activities have been suggested (1). The role of chemokines in tumor growth is still unclear, and some new evidence for angiogenic and angiostatic effects will be discussed (see *Angiogenesis*).

### *Selectivity*

Collectively, chemokines and chemokine receptors form a sophisticated system for the regulation of leukocyte and lymphocyte traffic across different compartments from the tissue of origin and the blood to sites of homing, host defense, or disposal. Substantial new information has been obtained about the selectivity and the complexity of the system, and some simplifications are no longer justified. The concept, for instance, that CXC chemokines act primarily on neutrophils, whereas CC chemokines act on the other types of leukocytes must be revised in view of the recent identification of new CXC chemokine receptors, CXCR3 and CXCR4, that mediate lymphocyte recruitment (61, 65, 71). When considering the ligands of the four CXC and five CC chemokine receptors (Table 1), an interesting difference becomes apparent: CXC chemokines have high affinity for single receptors (IL-8 which binds to CXCR1 and CXCR2 is the only exception), whereas most CC chemokines recognize two or more receptors

that differ in ligand specificity and cellular distribution. MCP-1 and eotaxin, which act via CCR2 and CCR3, respectively, are the only CC chemokines with restricted receptor usage. It is conceivable that CXC chemokines elicit more selective leukocyte responses than CC chemokines.

### *Tissue-Bound Chemokines*

The early observation that IL-8 is effective for several hours after intradermal application (142) suggested that chemokines can associate in active form with the tissue matrix. In vitro, IL-8 binds to glycosaminoglycans through its COOH-terminal  $\alpha$ -helix and remains active when complexed to heparin or heparan sulfate (143). To some degree, this interaction appears to be selective since IL-8, GRO $\alpha$ , NAP-2, and PF4 differ in their binding to heparin subfractions as shown by affinity co-electrophoresis (144). MIP-1 $\beta$  and RANTES also retain activity when bound to the tissue matrix and induce adherence of T cells (145, 146). Together these observations suggest that interaction with matrix glycosaminoglycans may help to confine chemokines within the site of their formation and so support the concept that the migration of leukocytes could be directed by a solid, rather than fluid, chemokine gradient. In situ binding of IL-8 and RANTES, but not of MIP-1 $\alpha$ , was observed in venular endothelial cells of the skin, and IL-8 was shown to bind to endothelia of mucosal and serosal sites, but not of parenchymatous tissues (147). It is possible that chemokines bound to the surface of endothelial cells direct diapedesis. The evidence for this attractive hypothesis, however, is still weak because only a few chemokines have been shown to bind, and the binding is restricted to some microvascular beds. In addition, chemokines, like other cationic proteins, can impair the activity of growth factors by competition for their binding sites on heparan sulfate (148, 149), a process that may explain some antiproliferative and angiostatic activities.

Most chemokines associate with the so-called Duffy antigen on erythrocytes (1). The "Duffy antigen receptor for chemokines" (DARC) was cloned and shown to consist of 338 amino acids and to comprise seven putative transmembrane domains. DARC has less than 20% amino acid identity with CXC and CC chemokine receptors and does not signal (150, 151). DARC is also expressed in some B and T cells, the endothelial cells of postcapillary venules and the Purkinje cells in the cerebellum (152). It is not clear, however, whether this promiscuous receptor is functionally relevant on endothelial cells and has a role in chemokine-dependent diapedesis because experiments with erythrocytes have shown that IL-8 is inactive toward neutrophils when bound to DARC (153).

### *Lymphocyte Recruitment*

Chemokines are now generally recognized as the long-sought mediators of lymphocyte recruitment. A first hint came from the early studies on IP10, which is

expressed at sites of lymphocyte accumulation in delayed-type hypersensitivity. Subsequent studies suggested a role for IL-8, but the evidence has repeatedly been questioned, and although papers in support of this concept are still being published, the CC chemokines have emerged as a major force in lymphocyte trafficking. RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  were shown several years ago to attract T lymphocytes, and, more recently, the monocyte chemotactic proteins were found to perform similar functions (see *The Monocyte Chemotactic Proteins*).

To define conditions for lymphocyte responsiveness, the migration induced by RANTES, MCP-1, and other CC chemokines was studied in relation to the expression of two main receptors, CCR1 and CCR2, in CD45RO<sup>+</sup> blood lymphocytes cultured under different stimulatory conditions (45). A close correlation between receptor expression and chemotaxis was observed and found to depend strictly on pretreatment of the cells with IL-2. Receptor expression and responsiveness are rapidly downregulated when IL-2 is withdrawn and are fully restored when IL-2 is supplied again. IL-2 can be substituted partially by IL-4, IL-10, or IL-12, but not by IL-13, IFN $\gamma$ , IL-1 $\beta$ , or TNF $\alpha$ . Interestingly, treatment with anti-CD3 alone or in combination with anti-CD28 rapidly downregulates receptors and migration. Receptor upregulation by IL-2 and downregulation by other stimuli of activation and proliferation suggest that T cells become responsive to chemokines after IL-2-mediated expansion and not during the early stages of antigen-dependent activation. Other studies showed that phytohemagglutinin-treated lymphocytes do not express CCR1 (88) and that human T cell clones lose migratory capacity after treatment with anti-CD3 (37). The opposite effect, however, was also reported (154).

The CC chemokines that attract lymphocytes are also chemotactic for monocytes, basophils, and eosinophils (2, 36), suggesting that the action of CC chemokines on lymphocytes is not selective. The role of MCP-1 in mononuclear cell recruitment was shown in a model of delayed-type hypersensitivity in rats (155) as well as in mice with *Cryptococcus neoformans* lung infection (156), where neutralizing antibodies against MCP-1 markedly decreased the local infiltration by monocytes and T lymphocytes.

A more selective recruitment of lymphocytes is likely to occur in response to IP10 and Mig, which bind to CXCR3, a receptor that does not recognize other chemokines and is confined to IL-2-activated T cells. Upon viral infection, for instance, IP10 and Mig are upregulated by locally produced IFN $\gamma$  and thus become available for the recruitment of effector lymphocytes as part of the antiviral response. The potential role of IFN $\gamma$ -induced chemoattractants in lymphocyte migration is highlighted by a comparison of the influx of radiolabeled neutrophils and lymphocytes in sheep after intradermal injection of chemoattractants and cytokines (157). The ratio of neutrophils to lymphocytes

was 45:1 after injection of IL-8 or C5a, about 5:1 after injection of TNF $\alpha$  or IL-1 $\alpha$ , and only 0.1:1 after injection of IFN $\gamma$ . Lymphotactin, a protein with only two cysteines and some structural similarity to chemokines, was also described as a selective attractant for lymphocytes (158). Such activities, however, have not been confirmed. Recombinant lymphotactin and two synthetic variants were extensively tested on human thymocytes and several preparations of T cells, including T cell clones, monocytes, and neutrophils, but no chemotactic activity nor  $[Ca^{2+}]_i$  changes were observed (159).

### *Angiogenesis.*

The study of the formation of new blood vessels was greatly encouraged by the recognition of the role of angiogenesis for tumor growth, and the effects of growth factors on endothelial cells (160). Popular models of angiogenesis are the neovascularization of the cornea or the chick chorioallantoic membrane. Angiogenic factors are applied locally in an adsorptive pellet, and angiostatic substances are either added to the pellet or injected systemically. Enhancement or inhibition of endothelial cell proliferation and/or in vitro migration are considered as predictive of angiogenic or angiostatic activity, respectively.

A possible involvement of chemokines in the regulation of angiogenesis was originally suggested by studies showing that PF4 has angiostatic (161) and potential anti-tumor activity (162). Similar effects were observed with other cationic proteins, and recently it was shown that PF4 and IP10 share binding sites on heparan sulfate and inhibit the proliferation of endothelial cells presumably by displacing growth factors (163). The opposite effect, angiogenesis, was reported for IL-8 and several other CXC chemokines with the NH<sub>2</sub>-terminal ELR motif (164, 165). Modification of the ELR motif reportedly confers angiostatic properties to IL-8, and introduction of the ELR motif converts the chemokine Mig from angiostatic to angiogenic. The mechanism of these phenomena has not been studied, and no receptors for angiogenic chemokines were described. It is unlikely that angiogenesis depends on neutrophil recruitment because some of the ELR-containing proteins studied, like platelet basic protein and connective tissue-activating peptide III, are inactive on neutrophils (1). In a later study the effects of IL-8 on human umbilical vein and dermal microvascular endothelial cells were examined, but no IL-8 binding nor IL-8-dependent  $[Ca^{2+}]_i$  changes were observed, and no CXCR1 or CXCR2 transcripts were detected by PCR (166).

Angiostatic rather than angiogenic activity by CXC chemokines was reported by Cao et al (167) who compared the GRO proteins. They found that GRO $\alpha$ , GRO $\beta$ , and PF4 inhibit the proliferation of capillary endothelial cells stimulated with basic fibroblast growth factor, whereas GRO $\gamma$  was inactive. In vivo GRO $\beta$  inhibited the neovascularization of the chorioallantoic membrane

and the mouse cornea, and it depressed the growth of murine Lewis lung carcinoma. Most recently, however, an anti-tumor effect was reported in SCID mice bearing human non-small cell lung cancer by neutralizing IL-8 with an antiserum (168). A tumor-promoting effect of IL-8 may also be inferred from the correlation between IL-8 expression and metastatic potential of melanoma cell lines in mice (169). On the other hand, IL-8 inhibits the proliferation of non-small cell cancer lines (170) and reduces tumorigenicity by recruitment of neutrophils in nude mice receiving tumor cells that express the human IL-8 gene (171). Anti-tumor activity was formerly observed in mice inoculated with tumor cells engineered to produce high levels of IP10, and the activity was found to depend on the recruitment of T lymphocytes and other white cells (172). Similar experiments were done with cells overexpressing murine MCP-1 (1). Furthermore, angiogenic properties were reported for soluble E-selectin and VCAM-1, which may be shed from the endothelial surface by enzymes released from adhering leukocytes (173). These and other observations emphasize the complexity of the pathophysiological process and suggest that, in many instances, the angiogenic effects of chemokines may be mediated by products released from the accumulating phagocytes.

#### *HIV Replication*

A most exciting new development came from the discovery that some chemokines function as HIV-1-suppressive factors. While searching for factors that delay the outbreak of AIDS, Cocchi et al (174) found that RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  produced by CD8<sup>+</sup> T cell lines are potent inhibitors of infection by monocyte/macrophage-tropic HIV-1 strains. These observations indicated that chemokines may determine the susceptibility to HIV infection and disease progression, and they suggested that chemokine receptors could in some way be involved in the recognition of HIV-1. Shortly thereafter, Feng et al (86) identified by expression cloning a 7TM receptor (termed fusin) that complements CD4 in a cell-fusion model of lymphocyte-tropic HIV-1 infection. Fusin is identical to CXCR4, and its ligand, SDF-1, was found to be a potent inhibitor of infection by lymphocyte-tropic HIV-1 strains in cell lines that coexpress CXCR4 and CD4 and in blood lymphocytes (65, 71). The HIV-suppressive factors RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  do not prevent the infection of cells expressing CXCR4. Unlike SDF-1, they interact with several receptors (see *CC Chemokine Receptors*) and one of them, CCR5, was shown by several groups to be the main coreceptor for entry of monocyte/macrophage-tropic HIV-1 strains (175–179). CCR3 and CCR2b have similar functions (176–178), but their role as HIV coreceptors is less prominent, suggesting that viral Env proteins have lower affinity for these receptors.

The repertoire of chemokine receptors in CD4<sup>+</sup> cells is likely to influence viral tropism, and it will be important to study the regulation of receptor

expression, particularly in T lymphocytes, macrophages, and dendritic cells. Viral entry is assumed to begin with the interaction of the highly variable viral Env protein, gp120, with CD4 and a chemokine receptor. Mutational changes of gp120 could lead to a switch in recognition from CCR5/CCR3 to CXCR4, and a shift from monocyte/macrophage-tropic to lymphocyte-tropic, syncytium-inducing strains. Recognition of CXCR4, which is widely expressed in blood leukocytes (66–70), could contribute to a spreading of the infection. There is already some evidence for a protective role of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  in individuals who remain uninfected despite high-risk exposure to HIV (180), and the therapeutic application of chemokines to prevent infection may be considered. Of particular interest is the possibility that SDF-1 in combination with CC chemokines could help to decrease virus load and prevent the emergence of the syncytium-inducing viruses characteristic for the progression to AIDS.

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## The Functional Role of the ELR Motif in CXC Chemokine-mediated Angiogenesis\*

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In this study, we demonstrate that the CXC family of chemokines displays disparate angiogenic activity depending upon the presence or absence of the ELR motif. CXC chemokines containing the ELR motif (ELR-CXC chemokines) were found to be potent angiogenic factors, inducing both *in vitro* endothelial chemotaxis and *in vivo* corneal neovascularization. In contrast, the CXC chemokines lacking the ELR motif, platelet factor 4, interferon  $\gamma$ -inducible protein 10, and monokine induced by  $\gamma$ -interferon, not only failed to induce significant *in vitro* endothelial cell chemotaxis or *in vivo* corneal neovascularization but were found to be potent angiostatic factors in the presence of either ELR-CXC chemokines or the unrelated angiogenic factor, basic fibroblast growth factor. Additionally, mutant interleukin-8 proteins lacking the ELR motif demonstrated potent angiostatic effects in the presence of either ELR-CXC chemokines or basic fibroblast growth factor. In contrast, a mutant of monokine induced by  $\gamma$ -interferon containing the ELR motif was found to induce *in vivo* angiogenic activity. These findings suggest a functional role of the ELR motif in determining the angiogenic or angiostatic potential of CXC chemokines, supporting the hypothesis that the net biological balance between angiogenic and angiostatic CXC chemokines may play an important role in regulating overall angiogenesis.

Angiogenesis, characterized by the neof ormation of blood vessels, is an essential biological event encountered in a number of physiological and pathological processes, such as embryonic development, the formation of inflammatory granulation tissue during wound healing, chronic inflammation, and the growth of malignant solid tumors (1–5). Neovascularization can be rapidly induced in response to diverse pathophysiological stimuli. Under conditions of homeostasis, the rate of capillary endothelial cell turn-over is typically measured in months or

years (6, 7). However, the process of angiogenesis during normal wound repair is rapid, transient, and tightly controlled. During neovascularization, normally quiescent endothelial cells are stimulated, degrade their basement membrane and proximal extracellular matrix, migrate directionally, divide, and organize into new functioning capillaries invested by a basal lamina (1–5). The abrupt termination of angiogenesis that accompanies the resolution of the wound repair suggests two possible mechanisms of control: a marked reduction in angiogenic mediators coupled with a simultaneous increase in the level of angiostatic factors that inhibit new vessel growth (8). In contrast to neovascularization of normal wound repair, tumorigenesis is associated with exaggerated angiogenesis, suggesting the existence of augmented angiogenic and reduced levels of angiostatic mediators (3, 9). Although most investigations studying angiogenesis have focused on the identification and mechanism of action of angiogenic factors, recent evidence suggests that angiostatic factors may play an equally important role in the control of neovascularization (8, 10–26).

Recently, platelet factor 4 (PF4),<sup>1</sup> a member of the CXC chemokine family, has been found to be an inhibitor of angiogenesis (27). In contrast, interleukin-8 (IL-8), another member of the CXC chemokine family, has been shown to have potent angiogenic properties (28–30). Although these CXC chemokines have significant homology on the amino acid level, one of the major differences between IL-8 and PF4 is the presence in IL-8 of the sequence Glu-Leu-Arg (the ELR motif), which is not found in PF4 (31–34). These three amino acids appear to be important in ligand/receptor interactions on neutrophils (35, 36) and are highly conserved in all members of the CXC chemokine family that demonstrate biological activation of neutrophils (35, 36).

In this study, we demonstrate that members of the CXC chemokine family that contain the ELR motif, as compared with members that lack these three amino acids, are potent inducers of angiogenic activity. In addition, we show that CXC chemokines that lack the ELR motif, PF4, interferon  $\gamma$ -inducible protein 10 (IP-10), and monokine induced by  $\gamma$ -interferon (MIG) are potent inhibitors of both CXC (ELR) chemokine and

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<sup>1</sup> The abbreviations used are: PF4, platelet factor 4; IL-8, interleukin-8; IP-10, interferon  $\gamma$ -inducible protein 10; MIG, monokine induced by  $\gamma$ -interferon; bFGF, basic fibroblast growth factor; PAGE, polyacrylamide gel electrophoresis; GRO, growth-related oncogene; ENA-78, epithelial neutrophil activating protein-78; GCP-2, granulocyte chemotactic protein-2; PBS, phosphate-buffered saline; GST, glutathione S-transferase; HPF, high power field(s); IFN, interferon.

basic fibroblast growth factor (bFGF)-induced angiogenesis. Moreover, substitution of the ELR motif in IL-8 generated proteins that antagonized the angiogenic effects of ELR-CXC chemokines and bFGF, while a mutant of MIG containing the ELR motif was angiogenic. These results suggest that the presence or absence of the ELR motif in CXC chemokines functionally defines the angiogenic (ELR containing) or angiostatic (non-ELR) characteristics of these proteins. These findings support the notion that CXC chemokines play an important role in the regulation of angiogenesis by acting as either angiogenic or angiostatic factors.

#### MATERIALS AND METHODS

**Reagents**—Human recombinant IP-10 (lyophilized protein with no additives) was purchased from Pepto Tech Inc. (Rocky Hill, NJ). IP-10 was >98% pure by SDS-PAGE analysis. Human recombinant bFGF (lyophilized protein with no additives) was purchased from R&D Systems Inc. (Minneapolis, MN). bFGF was >97% pure, as determined by NH<sub>2</sub> terminus analysis and SDS-PAGE. Recombinant human PF4, natural NH<sub>2</sub>-terminal truncated forms of platelet basic protein (connective tissue activating protein-III,  $\beta$ -thromboglobulin, and neutrophil activating protein-2), recombinant IL-8 (72 amino acids), recombinant human growth-related oncogene (GRO- $\alpha$ ), recombinant human GRO- $\beta$ , recombinant human GRO- $\gamma$ , and recombinant human epithelial neutrophil activating protein-78 (ENA-78) were provided by A. Walz. These chemokines were lyophilized proteins with no additives and were >97% pure, as determined by NH<sub>2</sub> terminus analysis and SDS-PAGE. Natural granulocyte chemotactic protein-2 (GCP-2; Ref. 34) was provided by J. Van Damme and was >98% pure, as determined by NH<sub>2</sub> terminus analysis and SDS-PAGE. Endotoxin levels were less than 0.1 ng/ $\mu$ g for the above cytokines. The proteins were either reconstituted in Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin for analysis in endothelial cell chemotaxis assays, Hanks' balanced salt solution with calcium/magnesium for analysis in neutrophil cell chemotaxis assays, or 1  $\times$  PBS for the corneal micropocket model of angiogenesis.

**Bacterial Host Strains and Vectors**—The *Escherichia coli* K12 strain DH5 $\alpha$ F' (Life Technologies, Inc.) was used as host for the propagation and maintenance of M13 DNA, and for expression of IL-8 and MIG proteins. Strain CJ236 was used to prepare uracil-DNA for use in site-directed mutagenesis (37). pGEX 4T-1 (Pharmacia Biotech Inc.) was used as the expression vector for all MIG cDNAs (38). pMAL-c2 (New England Biolabs) was used as the expression vector for all IL-8 cDNAs.

**Mutagenesis, Recombinant DNA, and Sequencing Protocols**—Site-directed mutagenesis was followed the protocol described by Kunkel *et al.* (37). Individual clones were sequenced using the dideoxynucleotide method (39) with modifications described in the Sequenase<sup>®</sup> (U.S. Biochemical Corp.) protocol. M13 (replicative form) DNA (40) containing confirmed MIG mutations was cleaved with *Bam*HI and *Xba*I (New England Biolabs) and subcloned into pGEX 4T-1. A 197-base pair *Sac*I (New England Biolabs) fragment from pMAL.hIL-8 (maltose binding protein-Ile-Glu-Gly-Arg-human IL-8 fusion protein expression vector) containing the coding sequence for the NH<sub>2</sub>-terminal 49 amino acids of the 72-amino acid form of human IL-8 sequence was subcloned to pUC118 (ATCC) digested with *Sac*I for site-directed mutagenesis. Clones containing confirmed IL-8 mutations were cleaved with *Sac*I and subcloned into pMAL.hIL-8 digested with *Sac*I.

**Cloning, Expression, and Purification of Human MIG**—The open reading frame of human MIG (38) was amplified from cDNA generated from interferon  $\gamma$ -stimulated (1000 units/ml for 16 h) THP-1 cells by polymerase chain reaction. The 5'-primer used, 5'-CAAGGTGGA-TCCATGAAGAAAAGTGGTGTTC-3', encodes a *Bam*HI restriction site immediately upstream of the ATG start site. The 3'-primer, 5'-GCAAGCTCTAGATTATGTAGTCTTCTTTGACGAGAACG-3', encodes a *Xba*I restriction site immediately downstream of the TAA stop codon. The 402-base pair fragment was subcloned to M13mp19 and was confirmed as the human MIG open reading frame by sequencing. Thr<sup>23</sup> of the open reading frame sequence is the predicted NH<sub>2</sub>-terminal amino acid of the mature, secreted MIG protein (38) and will be referred to here as amino acid position 1. Amino acids Lys<sup>6</sup> and Gly<sup>7</sup> were modified to Glu and Leu, respectively, by site-directed mutagenesis, generating the MIG mutant ELR-MIG. A *Bam*HI restriction site was introduced overlapping Gly<sup>-1</sup> and Thr<sup>1</sup> by site-directed mutagenesis (37), resulting in mutant MIG or ELR-MIG cDNAs encoding a Thr<sup>1</sup> to Ser substitution. 324-base pair fragments obtained from correct M13

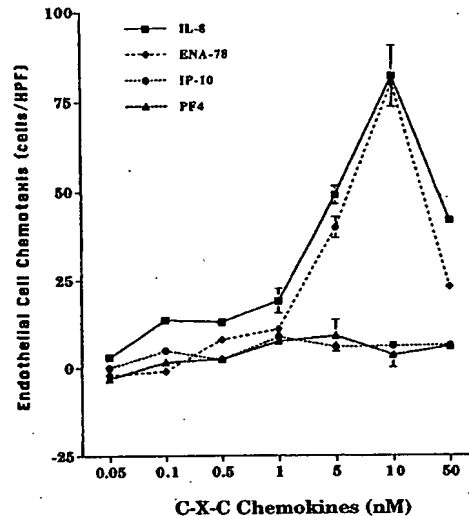


FIG. 1. Endothelial cell chemotaxis in response to CXC chemokines (50 pM to 50 nM). To demonstrate specific migration, background (unstimulated control) migration (cells/HPF) was subtracted.

TABLE I  
Endothelial cell chemotaxis in response to CXC chemokines  
Experimental  $n = 3$ .

Condition (10 nM)	Increase over control
	-fold
IL-8	5.1 $\pm$ 0.7
ENA-78	6.5 $\pm$ 0.8
GCP2	6.2 $\pm$ 0.4
GRO- $\alpha$	5.3 $\pm$ 0.3
GRO- $\beta$	3.5 $\pm$ 0.2
GRO- $\gamma$	4.5 $\pm$ 0.5
PBP	3.4 $\pm$ 0.1
CTAP-III	5.2 $\pm$ 0.3
$\beta$ -TG	1.6 $\pm$ 0.2
NAP-2	3.9 $\pm$ 0.1
IP-10	0.1 $\pm$ 0.1
PF4	0.1 $\pm$ 0.0
MIG	0.1 $\pm$ 0.0

RF clones digested with *Bam*HI/*Xba*I were subcloned to pGEX 4T-1 to generate glutathione S-transferase-MIG fusion DNAs (GST-MIG or GST-ELR-MIG). The sequence encoded by these DNAs contains the thrombin recognition sequence LVPRGS between the GST and MIG sequences. Digestion of GST-MIG fusion protein with thrombin is predicted to release MIG protein having an NH<sub>2</sub>-terminal sequence Gly-Ser-Pro, versus the predicted nonmodified NH<sub>2</sub>-terminal sequence Thr-Pro.

Cultures of *E. coli* strain DH5 $\alpha$ F' harboring GST-MIG or GST-ELR-MIG plasmid were grown in 1 liter of LB media containing 50  $\mu$ g/ml ampicillin to  $A_{600} \sim 0.5$  at 22  $^{\circ}$ C with aeration, and protein expression was induced by the addition of 0.1 mM final isopropyl-1-thio- $\beta$ -D-galactoside and continued incubation at 22  $^{\circ}$ C for 5–6 h. After induction, the cells were harvested by centrifuging at 6,000  $\times$  g for 10 min and the pellet was washed once in ice-cold PBS and resuspended in 10 ml of ice-cold 10 mM HEPES, 30 mM NaCl, 10 mM EDTA, 10 mM EGTA, 0.25% Tween 20, 1 mM phenylmethylsulfonyl fluoride (added fresh), pH 7.5 (lysis buffer). The resulting suspension was quick-frozen in liquid nitrogen. After thawing, phenylmethylsulfonyl fluoride was again added to yield a final concentration of 2 mM. The suspension was sonicated using a Branson Sonifier 250 equipped with a microtip for 2 min at output setting 5 with a 40% duty cycle. Triton X-100 was added to a final concentration of 1%, and the lysate was mutated for 30 min at room temperature to aid in the solubilization of the fusion protein. The lysate was then centrifuged at 34,500  $\times$  g for 10 min, and the supernatant was transferred to a fresh tube.

The GST-MIG protein was purified using the Pharmacia GST purification module (Pharmacia) essentially as described in the manufac-

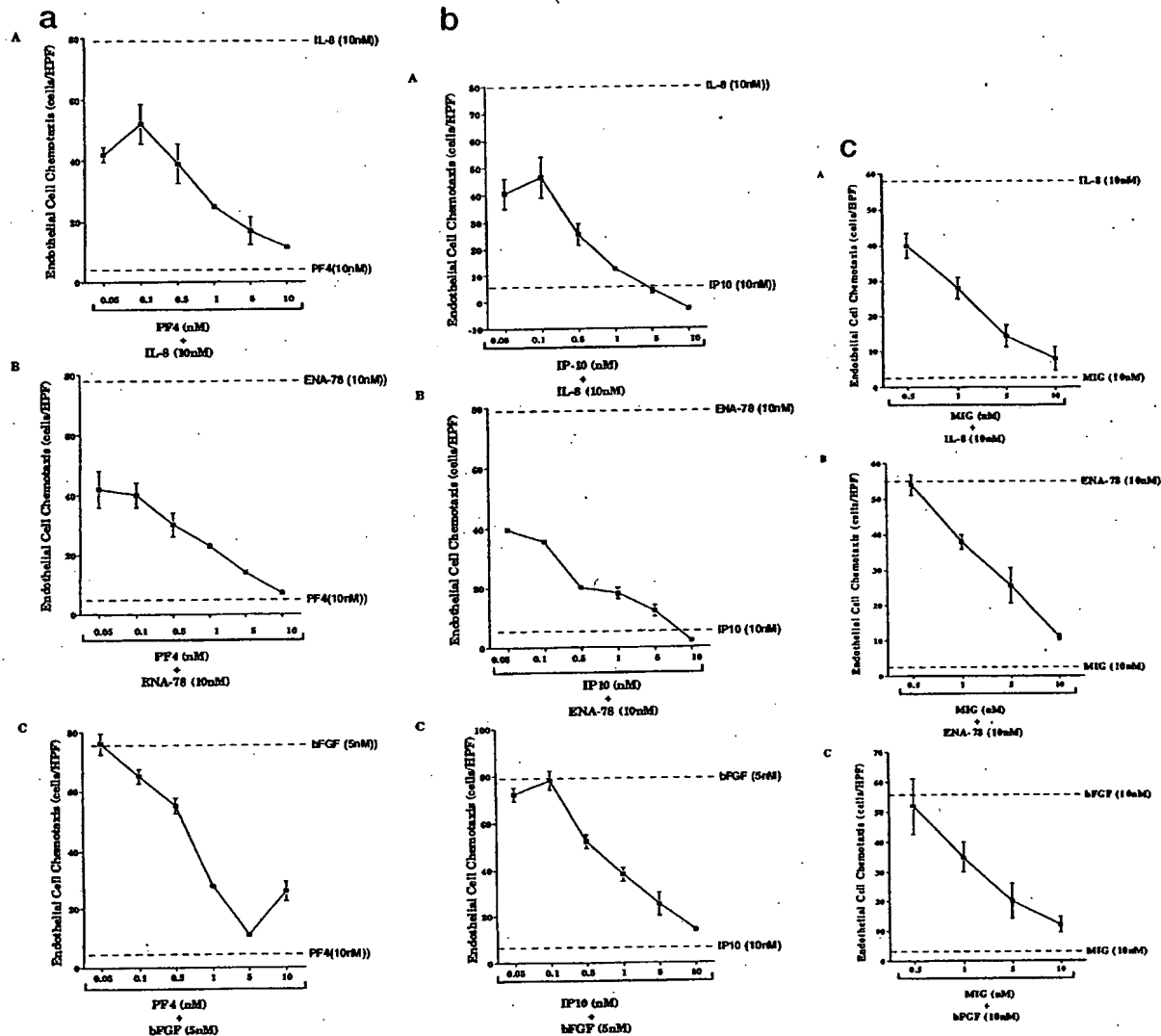


Fig. 2. Endothelial cell chemotaxis in response to IL-8 (10 nM), ENA-78 (10 nM), and bFGF (5 nM) in the presence of varying concentrations PF4 (50 pM to 10 nM; part a), IP-10 (50 pM to 10 nM; part b), and MIG (500 pM to 10 nM; part c). To demonstrate specific migration, background (unstimulated control) migration (cells/HPF) was subtracted.

turer's protocol. GST-fusion protein sonicate was passed over a 2-ml glutathione-Sepharose 4B column equilibrated in PBS. After washing with PBS, the GST-fusion protein was eluted with 3 column volumes of 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0. 10 units of thrombin/A<sub>250</sub> unit of fusion protein was added to the eluted GST-MIG or GST-ELR-MIG fusion protein and incubated at room temperature with occasional gentle mixing for 2–3 h. MIG or ELR-MIG protein was ≥95% cleaved from the GST protein under these conditions as monitored by SDS-PAGE (41). The pH of the MIG-containing solution was adjusted to 4.0 using 0.5 M sodium acetate, pH 4.0, filtered through a cellulose acetate 0.45-μm filter (Costar) and passed over a Mono S column (Pharmacia) equilibrated with 20 mM sodium acetate, pH 4.0. MIG protein was eluted as a single peak using a 0–2 M NaCl gradient, and dialyzed against 0.5 mM NaPO<sub>4</sub>, 20 mM NaCl, pH 7.0. Purified MIG and ELR-MIG was obtained endotoxin-free (<1.0 enzyme units/ml; QCL-1000 test, BioWhittaker), and yields ranged from 100–200 μg/liter (quantitated by amino acid analysis) with a purity of >95% (determined by SDS-PAGE, with apparent molecular mass of 16 kDa; amino acid analysis accuracy > 90%). Mass spectrometry of the purified MIG and ELR-MIG proteins confirmed their predicted mass.

**Cloning, Expression, and Purification of Human IL-8**—The 72-amino acid mature form of IL-8 was amplified using polymerase chain

reaction from an IL-8 cDNA in pET3a (kindly provided by I. U. Schraufstatter, Scripps Clinic). The 5'-primer used, 5'-AGTGCTAAAGAACTAGATG-3', encodes the beginning reading frame of IL-8, and the 3' primer, 5'-GGGATCCTCATGAATTCTC-3', contains a *Bam*HI restriction site immediately after the stop codon. The 220-base pair PCR product was purified by gel electrophoresis, digested with *Bam*HI (New England Biolabs), subcloned into pMal-c2 previously digested with *Xmn*I and *Bam*HI (New England Biolabs) to generate pMal.hIL-8. Clones containing inserts were confirmed by sequencing. Site-directed mutagenesis was used to modify amino acids Glu<sup>4</sup>-Leu<sup>5</sup>-Arg<sup>6</sup> to Thr-Val-Arg or Asp-Leu-Gln, generating TVR-IL-8 or DLQ-IL-8, respectively. Correct clones were identified by sequencing and subcloned as *Sac*I fragments from pUC118 into pMal.hIL-8 digested with *Sac*I.

Cultures of *E. coli* strain DH5aF' harboring pMal.hIL-8, pMal.TVR-IL-8, or pMal.DLQ-IL-8 were grown in 1-liter LB media containing 50 μg/ml ampicillin to A<sub>600</sub> ~ 0.5 at 37 °C with aeration, and protein expression was induced by the addition of 0.3 mM final isopropyl-1-thio-β-D-galactoside and continued incubation at 37 °C for 2 h. Cells were harvested by centrifuging at 5800 × g for 10 min and the pellet was washed once in ice-cold PBS and resuspended in 10 ml of ice-cold lysis buffer. The resulting suspension was quick-frozen in liquid nitrogen.

After thawing, the suspension was sonicated using a Branson Soni-

TABLE II  
The IC50 of PF4, IP-10, and MIG for the inhibition of the agonists  
IL-8, ENA-78, and bFGF

Experimental  $n = 3$ .

Agonist	IL-8 (10 nM)	ENA78 (10 nM)	bFGF (5 nM)
	$M$	$M$	$M$
PF4	$5 \times 10^{-11}$	$5 \times 10^{-11}$	$1 \times 10^{-9}$
IP-10	$5 \times 10^{-11}$	$5 \times 10^{-11}$	$1 \times 10^{-9}$
MIG	$5 \times 10^{-10}$	$5 \times 10^{-9}$	$1 \times 10^{-9}$

fier 250 equipped with a microtip for 2 min at output setting 5 with a 40% duty cycle. The suspension was clarified by centrifugation at 9000  $\times g$ , the supernatant was diluted 5-fold in 10 mM NaPO<sub>4</sub>, 500 mM NaCl, 1 mM EGTA, 0.25% Tween 20, pH 7.0 (column buffer), and loaded onto a 10-ml amylose resin (New England Biolabs) affinity column. After extensive washing with column buffer, the maltose binding protein fusion protein was eluted with column buffer containing 10 mM maltose. Mutein or wild-type IL-8 proteins were released by incubation with 1  $\mu$ g of Factor Xa (New England Biolabs)/A<sub>280</sub> maltose binding protein fusion protein at room temperature overnight and were then passed over a Mono S column (Pharmacia) equilibrated in 10 mM NaPO<sub>4</sub>, pH 6.2, and eluted in a 0–1 M NaCl gradient. 1 ml of amylose resin was added to fractions containing mutant or wild-type IL-8 protein to remove residual free maltose binding protein by incubation for 30 min at room temperature with gentle shaking. The resin was removed by centrifugation, and the supernatant was dialyzed against 0.5 mM NaPO<sub>4</sub>, 20 mM NaCl, pH 7.5. Yields were ranged from 0.2 to 3.5 mg for wild-type or mutant IL-8 proteins and were  $\geq 95\%$  pure as assessed by SDS-PAGE and endotoxin-free ( $<1.0$  enzyme units/ml). Proteins were quantitated by amino acid analysis, and had accuracies between 88–93%.

**Endothelial Cell Chemotaxis**—Endothelial cell chemotaxis was performed in 48-well chemotaxis chambers (Nucleopore Corp.) as described previously (28, 42). Briefly, bovine adrenal gland capillary endothelial cells were suspended at a concentration of  $10^6$  cells/ml in Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin and placed into each of the bottom wells (25  $\mu$ l). Nucleopore chemotaxis membranes (5- $\mu$ m pore size) were coated with 0.1 mg/ml gelatin. The membranes were placed over the wells and the chambers were sealed, inverted, and incubated for 2 h to allow cells to adhere to the membrane. The chambers were then reinverted; 50  $\mu$ l of sample (containing media alone, ELR-CXC chemokines, non-ELR-CXC chemokines, bFGF, or combinations of ELR-CXC and non-ELR-CXC chemokines or non-ELR-CXC chemokines and bFGF) was dispensed into the top wells and reincubated for an additional 2 h. Membranes were then fixed and stained with Diff-Quik staining kit (American Scientific Products), and cells that had migrated through the membrane were counted in 10 high power fields (HPF; 400 $\times$ ). Results were expressed as the number of endothelial cells that migrated per HPF after subtracting the background (unstimulated control) to demonstrate specific migration. Each sample was assessed in triplicate. Experiments were repeated at least three times.

**Neutrophil Chemotaxis**—Heparinized venous blood was collected from healthy volunteers and mixed 1:1 with 0.9% saline, and mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Human neutrophils were then isolated by sedimentation in 5% dextran, 0.9% saline (Sigma) and separated from erythrocytes by hypotonic lysis. After washing twice, neutrophils were suspended in Hanks' balanced salt solution with calcium/magnesium (Life Technologies, Inc.) at a concentration of  $2 \times 10^6$  cells/ml. Neutrophils were  $>95\%$  viable as determined by trypan blue exclusion. Neutrophil chemotaxis was performed as described previously (43, 44). 150  $\mu$ l of sample (ELR-CXC, non-ELR-CXC, or combination of ELR-CXC and non-ELR-CXC chemokines),  $1 \times 10^{-7}$  M formylmethionyleucylphenylalanine (Sigma), or Hanks' balanced salt solution (Life Technologies, Inc., Grand Island, NY) alone were placed in duplicate bottom wells of a blind well chemotaxis chamber. A 3- $\mu$ m pore size polycarbonate filter (polyvinylpyrrolidone-free, Nucleopore Corp.) was placed in the assembly, and 250  $\mu$ l of human neutrophil suspension was placed in each of the top wells. Chemotaxis chamber assemblies were incubated at 37  $^{\circ}$ C in humidified 95% air, 5% CO<sub>2</sub> for 60 min. The filters were removed, fixed in absolute methanol, and stained with 2% toluidine blue (Sigma). Neutrophils that had migrated through to the bottom of the filter were counted in 10 HPF (400 $\times$ ) using a Javelin chromachip camera (Javelin Electronics, Japan) attached to a Olympus BH-2 microscope interfaced with a Macintosh II computer containing an Image Capture 1000 frame grabber (Scion Corp., Walkersville, MD) and NIH Image, version 1.40 software (Na-

TABLE III  
Neutrophil chemotaxis in response to CXC chemokines. Control is media alone

Experimental  $n = 3$ .

Condition (10 nM)	Cells/HPF
Control	$18.4 \pm 1.8$
IP10	$20.7 \pm 4.2$
MIG	$8.6 \pm 1.5$
IL-8	$96.4 \pm 6.5$
IL-8 + IP10	$94.1 \pm 9.3$
IL-8 + MIG	$78.6 \pm 10.5$

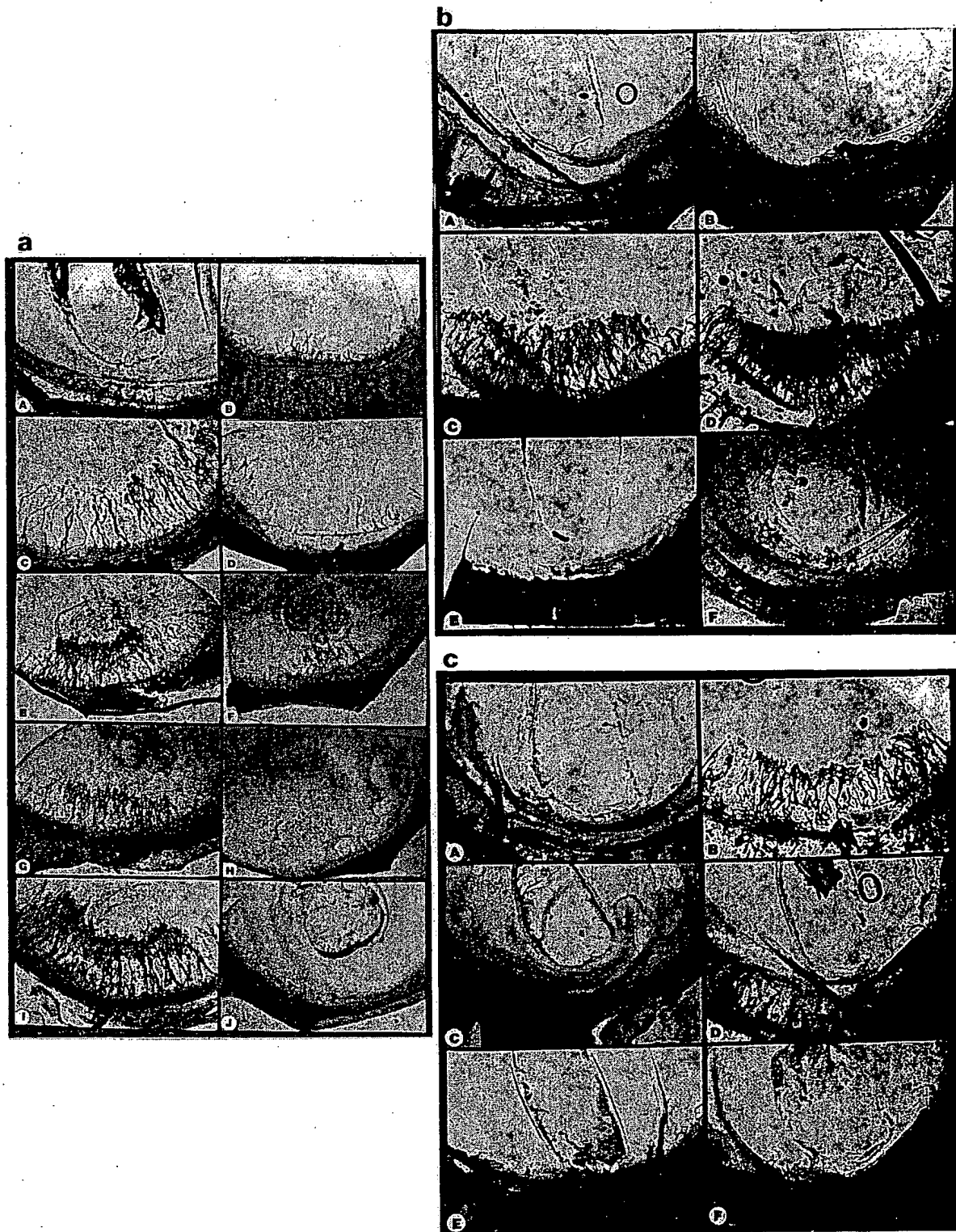
tional Institutes of Health Public Software, Bethesda, MD). Each sample was assessed in triplicate. Experiments were repeated at least three times.

**Corneal Micropocket Model of Angiogenesis**—*In vivo* angiogenic activity was assayed in the avascular cornea of Long Evans rat eyes, as described previously (28, 29, 42). Briefly, cytokines were combined with sterile Hydron (Interferon Sciences Inc.) casting solution, and 5- $\mu$ l aliquots were air-dried on the surface of polypropylene tubes. Prior to implantation, pellets were rehydrated with normal saline. Animals were anesthetized with an intraperitoneal injection of ketamine (150 mg/kg) and atropine (250  $\mu$ g/kg). Rat corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1–2 mm from the limbus). 6 days after implantation, animals were pretreated intraperitoneally with 1000 units of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ), anesthetized with ketamine (150 mg/kg), and perfused with 10 ml of colloidal carbon via the left ventricle. Corneas were then harvested and photographed. No inflammatory response was observed in any of the corneas treated with the above cytokines. Positive neovascularization responses were recorded only if sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were observed. Negative responses were recorded when either no growth was observed or when only an occasional sprout or hairpin loop displaying no evidence of sustained growth was detected.

**Statistical Analysis**—Data were analyzed by a Macintosh IIx computer using the Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA). Data were expressed as mean  $\pm$  S.E. and compared using the nonparametric analysis with the Wilcoxon signed rank test. Data were considered statistically significant if  $p$  values were  $\leq 0.05$ .

## RESULTS

**CXC Chemokines Display Disparate Angiogenic Activity**—Endothelial cell chemotaxis was performed in the presence or absence of IL-8, ENA-78, PF4, and IP-10 at concentrations of 50 pM to 50 nM. Both IL-8 and ENA-78 demonstrated a dose-dependent increase in endothelial migration that was significantly greater ( $p < 0.05$ ) than control (background) at concentrations equal to or above 0.1 and 1 nM, respectively, with evidence of a "bell-shape" curve seen with other chemotactic factors (Fig. 1). In contrast, neither PF4 nor IP-10 induced significant ( $p > 0.05$ ) endothelial cell chemotaxis (Fig. 1). Similar findings were also observed using either human umbilical or dermal microvascular endothelial cells (data not shown). The migration seen in response to IL-8 or ENA-78 was due to chemotaxis, not chemokinesis, as checkerboard analysis demonstrated directed, not random, migration (data not shown). Other CXC chemokines were tested for their ability to induce endothelial cell chemotaxis, including ELR-CXC chemokines IL-8, ENA-78, GCP-2, GRO- $\alpha$ , GRO- $\beta$ , GRO- $\gamma$ , platelet basic protein, connective tissue activating protein-III, and neutrophil-activating protein-2 or the non-ELR CXC chemokines IP-10, PF4, and MIG (Table I). In a similar fashion to IL-8 or ENA-78, all of the ELR-CXC chemokines tested demonstrated significant ( $p < 0.05$ ) endothelial cell chemotactic activity over the background control, whereas the endothelial cell chemotactic activity induced by MIG was either similar to background control or to the endothelial cell chemotactic activity seen with either PF4 or IP-10. These findings suggested that CXC chemokines could be divided into two groups with defined biolog-



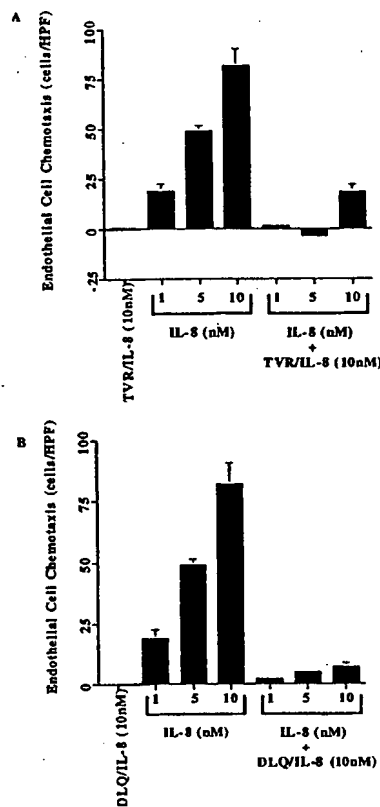
**Fig. 3.** Rat cornea neovascularization in response to ELR-CXC chemokines, non-ELR-CXC chemokines, bFGF, or combinations of these cytokines. *Part a*, panels *A*, *B*, *C*, *E*, *G*, and *I*, respectively, represent the corneal neovascular response to a hydron pellet alone (vehicle control), IP-10 (10 nM), IL-8 (10 nM), ENA-78 (10 nM), GRO- $\alpha$  (10 nM), or GCP-2 (10 nM); *part a*, panels *D*, *F*, *H*, and *J*, respectively, represent the combination of IL-8 with IP-10, ENA-78 with IP-10, GRO- $\alpha$  with IP-10, or GCP-2 with IP-10. *Part b*, panels *A*–*D*, respectively, represent the corneal neovascular response to a hydron pellet alone (vehicle control), MIG (10 nM), IL-8 (10 nM), or ENA-78 (10 nM); *part b*, panels *E* and *F*, respectively,

ical activities, one that contains the ELR motif and is chemotactic for endothelial cells and the other that lacks the ELR motif and does not induce endothelial chemotaxis.

**PF4, IP-10, or MIG Inhibit IL-8, ENA-78, or bFGF-induced Angiogenic Activity**—While the above experiments suggested that PF4, IP-10, and MIG were not significant chemotactic factors for endothelial cells, we postulated that these CXC chemokines may be potent inhibitors of angiogenesis. To test this hypothesis, endothelial cell chemotaxis was performed in the presence or absence of IL-8 (10 nM), ENA-78 (10 nM), or bFGF (5 nM) with or without varying concentrations of PF4, IP-10, or MIG from 0 to 10 nM (Fig. 2, *a–c*, respectively). Endothelial cell migration in response to either IL-8, ENA-78, or bFGF was significantly inhibited by PF4, IP-10, or MIG in a dose-dependent manner (Fig. 2). PF4 and IP-10 in a concentration of 50 pM inhibited either IL-8- or ENA-78-induced endothelial chemotaxis by 50%, whereas, PF4 and IP-10 in a concentration of 1 nM attenuated the response to bFGF by 50% (Fig. 2, *a* and *b*, and Table II). MIG at a concentration of 1, 5, and 1 nM inhibited the endothelial cell chemotactic response to IL-8, ENA-78, and bFGF, respectively, by 50% (Fig. 2*c* and Table II). Interestingly, while IP-10 and MIG inhibited IL-8-induced endothelial cell chemotactic activity, neither IP-10 nor MIG were effective in attenuating IL-8-induced neutrophil chemotactic activity ( $p > 0.05$ ) (Table III).

The rat corneal micropocket model of neovascularization was used to determine whether IP-10 or MIG could inhibit the angiogenic activity of either the ELR-containing CXC chemokines or bFGF *in vivo*. Hydron pellets alone, pellets containing IL-8, ENA-78, GRO- $\alpha$ , GCP-2, IP-10, MIG, or bFGF in a concentration of 10 nM, or pellets containing combinations of 10 nM each of IL-8 + IP-10, ENA-78 + IP-10, GRO- $\alpha$  + IP-10, GCP-2 + IP-10, IL-8 + MIG, ENA-78 + MIG, bFGF + IP-10, or bFGF + MIG were embedded into the normally avascular rat cornea and assessed for a neovascular response (Fig. 3, *a–c*). The CXC chemokines (IL-8, ENA-78, GRO- $\alpha$ , or GCP-2) or bFGF-induced positive corneal angiogenic responses in six of six corneas, without evidence for significant leukocyte infiltration (assessed by light microscopy). In contrast, hydron pellets alone ( $n = 6$  corneas) or pellets containing either IP-10 or MIG (10 nM) ( $n = 6$  corneas for each chemokine) only resulted in a positive neovascular response in less than one of six corneas tested for each variable. When IP-10 was added in combination with the ELR-CXC chemokines (IL-8, ENA-78, GRO- $\alpha$ , or GCP-2) or bFGF (Fig. 3, *a* and *c*, respectively), IP-10 significantly abrogated the ELR-CXC chemokine and bFGF-induced angiogenic activity in five of six corneas ( $n = 6$  corneas for each manipulation). In addition, MIG inhibited IL-8, ENA-78, and bFGF-induced corneal angiogenic activity in a similar manner to IP-10 (Fig. 3, *b* and *c*).

**ELR Muteins of IL-8 and MIG Generate Angiostatic and Angiogenic Proteins, Respectively**—Muteins of IL-8 lacking the ELR motif and a mutant of MIG containing the ELR motif were generated to delineate its functional role in CXC chemokine-induced angiogenesis. The ELR motif in wild-type IL-8 was mutated to either TVR (TVR-IL-8; corresponding IP-10 sequence) or DLQ (DLQ-IL-8; corresponding PF4 sequence) by site-directed mutagenesis and expressed in *E. coli*. TVR-IL-8 and DLQ-IL-8 alone failed to induce endothelial cell chemotactic activity (Fig. 4, *A* and *B*, respectively), yet these muteins inhibited the maximal endothelial chemotactic activity of wild-



**FIG. 4.** Endothelial cell chemotaxis in response to the presence or absence of varying concentrations of IL-8 and IL-8 muteins, TVR-IL-8, and DLQ-IL-8. *Panel A* is endothelial cell chemotaxis in response to the presence or absence of varying concentrations of IL-8 (1–10 nM), TVR-IL-8 (10 nM), or in combination of varying concentrations of IL-8 with TVR-IL-8 (10 nM). *Panel B* is endothelial cell chemotaxis in response to the presence or absence of varying concentrations of IL-8 (1–10 nM), DLQ-IL-8 (10 nM), or in combination of varying concentrations of IL-8 with DLQ-IL-8 (10 nM). To demonstrate specific migration, background (unstimulated control) migration (cells/HPF) was subtracted.

type IL-8 by 83 and 88% ( $p < 0.05$ ), respectively (Fig. 4, *A* and *B*). Endothelial cell viability, as determined by the exclusion of trypan blue, was unchanged in the presence or absence of either of the IL-8 muteins (data not shown). Neither TVR-IL-8 nor DLQ-IL-8 induced neutrophil chemotaxis, nor were they effective in attenuating neutrophil chemotaxis in response to IL-8 (data not shown).

Using the *in vivo* rat cornea micropocket model of neovascularization, TVR-IL-8 (10 nM) alone did not induce a positive neovascular response in any of the six corneas tested. However, TVR-IL-8 (10 nM) in combination with either IL-8 (10 nM) or ENA-78 (10 nM) resulted in 83% reduction (only one of six corneas positive) in the ability of either IL-8 or ENA-78 to induce cornea neovascularization, as compared with 100% (six of six) of the corneas positive in the presence of either IL-8 or ENA-78 alone (Fig. 5). Moreover, the angiostatic activity of the IL-8 muteins was not only unique to inhibition of ELR-CXC chemokine-induced angiogenic activity, as TVR-IL-8 (10 nM)

represents the corneal neovascular response to the combination of IL-8 with MIG or ENA-78 with MIG. *Part c*, panels *A–D*, respectively, represents the corneal neovascular response to a hydron pellet alone (vehicle control), bFGF (5 nM), MIG (10 nM), or IP-10 (10 nM); *part c*, panels *E* and *F*, respectively, represents the corneal neovascular response to the combination of bFGF with MIG or bFGF and IP-10. All panels are at 25 $\times$  magnification.

**FIG. 5.** Rat cornea neovascularization in response to the IL-8, ENA-78, the IL-8 mutein (TVR-IL-8), and combinations of ENA-78 and TVR-IL-8 or IL-8 and TVR-IL-8. Panels A-D represent a hydron pellet alone, TVR-IL-8 (10 nM), ENA-78 (10 nM), and IL-8 (10 nM), respectively. Panels E and F represent the combination of ENA-78 and TVR-IL-8 and of IL-8 and TVR-IL-8, respectively. All panels are at 25 $\times$  magnification.



inhibited both bFGF-induced (10 nM) maximal endothelial cell chemotaxis by 65% ( $p < 0.05$ ) (Fig. 6a) and corneal neovascularization (five of six corneas;  $n = 6$  corneas for each cytokine) (Fig. 6b). Endothelial cell viability, as determined by the exclusion of trypan blue, was unchanged in the presence or absence of the TVR-IL-8 mutant (data not shown). In addition, ELR-MIG (10 nM) induced angiogenic responses in 8 of 10 corneas, as compared with wild-type MIG, which induced an angiogenic response in only 1 of 7 corneas (Fig. 7, A-D). Interestingly, MIG (10 nM) inhibited the angiogenic response of ELR-MIG in five of six corneas (Fig. 7, E and F). These data further support the importance of the ELR motif as a domain for mediating angiogenic activity. Similar to the synthetic ELR-IP-10 (36), ELR-MIG in a concentration of 10 pM to 100 nM failed to induce neutrophil chemotaxis (data not shown).

#### DISCUSSION

The CXC chemokine family of chemotactic cytokines are polypeptide molecules that appear, in general, to have proinflammatory activities. In monomeric forms, they range from 7 to 10 kDa and are characteristically basic heparin-binding proteins. They display four highly conserved cysteine amino acid residues with the first two cysteines separated by a non-conserved amino acid residue (the CXC cysteine motif). The CXC chemokines are all clustered on human chromosome 4 (q12-q21), and exhibit between 20 and 50% homology on the amino acid level (31-34). Over the last 2 decades, several human CXC chemokines have been identified, including PF4, NH<sub>2</sub>-terminal truncated forms of platelet basic protein (connective tissue activating protein-III,  $\beta$ -thromboglobulin, neutrophil-activating protein-2), IL-8, GRO- $\alpha$ , GRO- $\beta$ , GRO- $\gamma$ , ENA-78, GCP-2, IP-10, and MIG (31-34, 38). The ubiquitous nature

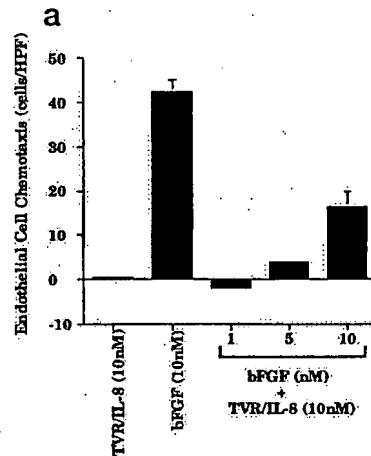
of CXC chemokine production by a variety of cells suggest that these cytokines may play a role in mediating biological events other than leukocyte chemotaxis.

We hypothesized that members of the CXC chemokine family may exert disparate effects in mediating angiogenesis as a function of the presence or absence of the ELR motif for primarily four reasons. First, members of the CXC chemokine family that display binding and activation of neutrophils share the highly conserved ELR motif that immediately precedes the first cysteine amino acid residue, whereas, PF4, IP-10, and MIG lack this motif (35, 36). Second, IL-8 (contains ELR motif) mediates both endothelial cell chemotactic and proliferative activity *in vitro* and angiogenic activity *in vivo* (28), and, in addition, endogenous IL-8 has been found to represent a major angiogenic factor that mediates net angiogenic activity of human nonsmall cell lung cancer (42). In contrast, PF4 (lacking the ELR motif) has been shown to have angiostatic properties (27), and attenuates growth of tumors *in vivo* (45). Third, the interferons (IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ ) are all known inhibitors of wound repair, especially angiogenesis (18, 46-49). These cytokines, however, up-regulate IP-10 and MIG from a number of cells, including keratinocytes, fibroblasts, endothelial cells, and mononuclear phagocytes (38, 50). Finally, we and others have found that IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  are potent inhibitors of the production of monocyte-derived IL-8, GRO- $\alpha$ , and ENA-78 (51, 52), supporting the notion that IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  may shift the biological balance of ELR- and non-ELR-CXC chemokines toward a preponderance of angiostatic (non-ELR) CXC chemokines.

In this study, we demonstrated that the members of the CXC chemokine family behave as either angiogenic or angiostatic



FIG. 6. Endothelial chemotaxis (part a) and rat cornea neovascularization (part b) in response to the presence or absence of varying concentrations of bFGF and the IL-8 mutein, TVR-IL-8 (10 nM). Part a is the endothelial chemotaxis in response to the presence or absence of varying concentrations of bFGF (1–10 nM), TVR-IL-8 (10 nM), or in combination of varying concentrations of IL-8 with TVR-IL-8 (10 nM). To demonstrate specific migration, background (unstimulated control) migration (cells/HPF) was subtracted. Part b, panels A–C is rat cornea neovascularization in response to bFGF (10 nM), TVR-IL-8 (10 nM), and the combination of bFGF and TVR-IL-8 at 25 $\times$  magnification, respectively.



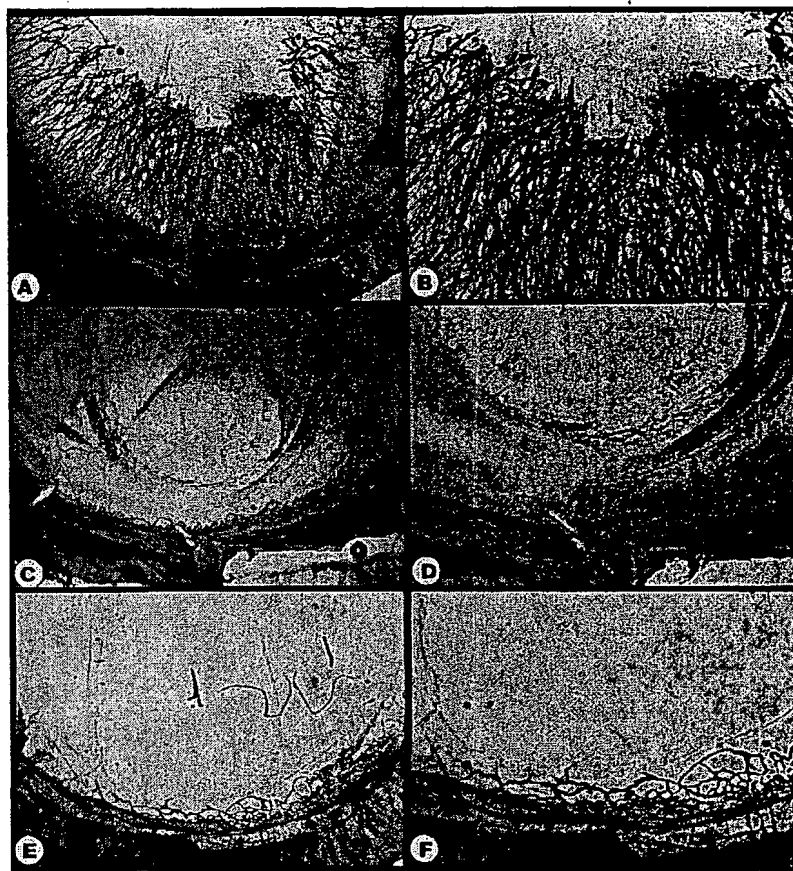
factors, depending upon the presence or absence of the ELR motif, respectively. This was supported using both *in vitro* (endothelial cell chemotaxis) and *in vivo* (rat cornea neovascularization) analyses. The evidence *in vitro* of directed (chemotaxis not chemokinesis by checkerboard analysis) migration in response to varying concentrations of ELR-CXC chemokines, IL-8, ENA-78, and the MIG mutein ELR-MIG, and the absence *in vivo* of leukocyte infiltration in the rat cornea during ELR-CXC chemokine-induced neovascularization, supports the direct role ELR-containing CXC chemokines play in mediating angiogenic activity. In contrast, CXC chemokines lacking the ELR motif, PF4, IP-10, MIG, and the two IL-8 muteins DLQ-IL-8 and TVR-IL-8, behave as potent angiostatic regulators of neovascularization, inhibiting not only the angiogenic activity of ELR-CXC chemokines, but also the structurally unrelated angiogenic factor, bFGF. Thus, the ELR motif appears to be essential for dictating the angiogenic activity of the CXC chemokines.

These findings are compatible with the ability of ELR-containing CXC chemokines to bind to both endothelial cells and neutrophils. However, the non-ELR muteins of wild-type IL-8, as well as IP-10 and MIG, inhibited ELR-CXC chemokine-induced angiogenesis but not neutrophil chemotaxis. The finding that IP-10 and MIG block other ELR-CXC chemokine-induced functions, *i.e.* angiogenesis, is unprecedented (53). Moreover, the muteins of wild-type IL-8, as well as IP-10 and MIG, also inhibited the angiogenic activity of the unrelated cytokine, bFGF, suggesting that a receptor system(s) other

than the IL-8 receptor may be operative on endothelial cells, which allows the angiostatic CXC chemokines to regulate both ELR-CXC chemokine and bFGF-induced angiogenic activity. This contention is further supported with the evidence that equimolar concentrations of mutant and wild-type IL-8 do not result in a 50% restoration of the endothelial cell chemotactic effect. This response is most likely due to the use of another "receptor" by the angiostatic CXC chemokines. While the Duffy antigen receptor for chemokines has been identified on post-capillary venule endothelial cells (54), this receptor binds not only ELR-CXC chemokines, but also MCP-1 and RANTES (55). We have found that these latter two CC chemokines are not chemotactic for endothelial cells (data not shown).

While the NH<sub>2</sub>-terminal ELR motif appears to be essential for angiogenic activity of CXC chemokines, it is uncertain whether the angiostatic properties of the non-ELR-CXC chemokines tested are due to the absence of the ELR motif. In particular, bFGF binds to low affinity cell surface receptors on endothelia that appear to be sulfate-proteoglycans (47, 56), and IL-8 specific binding to endothelial cells can be inhibited by preincubation with either heparin or heparan sulfate (57). One can speculate that, in the absence of the ELR motif, a potential mechanism exists by which another amino acid domain, perhaps within the COOH terminus of PF4, IP-10, MIG, TVR-IL-8, and DLQ-IL-8 may compete with either ELR-CXC chemokines or bFGF for proteoglycan binding sites and thus prevent endothelial cell activation and angiogenesis. It also possible, however, that the angiostatic effects of CXC chemokines lacking

**FIG. 7. Rat cornea neovascularization in response to the MIG mutein, ELR-MIG, MIG, and the combination of ELR-MIG and MIG. Panels A and B** represents the cornea neovascular response to ELR-MIG (10 nm) at 25 and 50 $\times$ , respectively. **Panels C and D** represent the cornea neovascular response to MIG (10 nm) at 25 and 50 $\times$  magnification, respectively. **Panels E and F** represent the cornea neovascular response to the combination of ELR-MIG and MIG at 25 and 50 $\times$  magnification, respectively.



the ELR motif are not directly competitive in nature, but are rather mediated through an independent receptor system. Studies in our laboratories are currently addressing these issues.

The interferons have been shown to inhibit wound repair and tumorigenesis through a presumed antiproliferative and angiostatic mechanism (46–49). While the expression of IL-8, GRO- $\alpha$ , and ENA-78 can be induced by a variety of factors, including TNF and IL-1, these chemokines are down-regulated by IFN- $\gamma$  (51, 52). In contrast, IP-10 and MIG expression is up-regulated by IFN- $\gamma$  (38, 50). This suggests that the disparate activity of the CXC chemokines as angiogenic or angiostatic factors may be physiologically relevant. The finding that IP-10 and MIG are potent angiostatic factors suggests that IFN- $\gamma$ , in part, may mediate its angiostatic activity through the local stimulation of production of IP-10 and MIG and by down-regulation of the expression of the angiogenic CXC chemokines, such as IL-8 and ENA-78. This suggests that the magnitude of local IFN- $\gamma$  expression by mononuclear cells during wound repair, chronic inflammation, or tumorigenesis may be a pivotal event in regulating both angiogenic (through negative feedback) and angiostatic (through positive feedback) CXC chemokine production.

Thus, our findings suggest that the ELR motif is the functional domain that dictates the angiogenic activity of the CXC chemokines, and supports the contention that members of the CXC chemokine family may exert disparate effects in mediating angiogenesis. The magnitude of the expression and relative concentrations of either angiogenic or angiostatic CXC chemokines during neovascularization may thus significantly con-

tribute to the regulation of net angiogenesis during either wound repair, chronic inflammation, or tumorigenesis.

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## Simple Method for Quantitation of Enhanced Vascular Permeability<sup>1</sup> (34695)

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The wall of certain vessels (venules and capillaries) of the microcirculation represent the blood-tissue barrier. This barrier is freely permeable to water, electrolytes, and small molecules, but only slightly permeable to proteins. The term "increased vascular permeability" refers to an alteration of this barrier, leading to an accelerated rate of passage of plasma proteins into the extravascular tissues: exudation. This phenomenon leads to swelling, which is one of the cardinal features of acute inflammation. Exudation is closely linked to other vascular phenomena, such as hyperemia and stasis. It has been generally accepted that some vital dyes, such as Evans blue, trypan blue or pontamine sky blue, given intravenously, become bound to plasma proteins, particularly to albumin. Therefore, the accumulation of such dyes in inflammatory lesions indicates exudation of plasma proteins. However, evaluation of experimental results in such tests often lacks precision. The present paper describes a simple physicochemical assay for the quantitative measurement of enhanced vascular permeability.

**Material and Methods.** Adult male albino rabbits, both male and female albino guinea pigs and female Wistar rats were used.

**Dye extraction method.** Evans blue was injected intravenously in concentrations of 60 mg/kg for rabbits and rats and 20 mg/kg for guinea pigs, respectively. Inflammatory

skin lesions were produced by intradermal injections of various inflammatory agents. The skin lesions were punched out with a standard steel punch (1.5–2.5 cm in diameter). To each piece of skin containing the lesion, 4.0 ml of formamide (Fisher Scientific Co. Ltd.) was added and incubated at 45° for 72–96 hr or at 65° for 24–36 hr. If necessary, the incubation time was prolonged, until the blue color of the skin completely disappeared. After filtration with glass filter (Pyrex, coarse; 1.0 cm in diameter), the optical density of the filtrate was measured at 620 m $\mu$  in a Zeiss PMQ II spectrophotometer. The total amount of dye can be calculated by means of a standard calibration curve.

**Simultaneous radioassay and dye extraction.** Evans blue (doses as above) were injected intravenously mixed with <sup>125</sup>RISA (radioiodinated human serum albumin; Charles Frosst and Co., Montreal, Canada). The ratio of <sup>125</sup>RISA to Evans blue was 1  $\mu$ Ci/mg for studies in rabbits and guinea pigs and  $\frac{1}{3}$   $\mu$ g/mg for experiments in rats. The punched out pieces of skin, containing the lesion, were placed in tubes containing 4 ml of formamide. First the radioactivity was measured in a  $\gamma$ -scaler (Model 4204 Nuclear Chicago), calibrated with cesium (44,000 counts  $\pm$  500/min) and known amounts of <sup>125</sup>RISA. Subsequently, the Evans blue was extracted and the amount of dye was determined as described above.

**Experiments to test vascular permeability.**

As known chemical mediators, synthetic bradykinin (Sandoz, Montreal, Canada), histamine (histamine base, Fisher Scientific Co., Toronto, Canada) and serotonin (serotonin sulfate, Upjohn Co., Kalamazoo, Michi-

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gan) were selected. Each of them was suspended in buffered (pH 7.4) saline at the following concentrations: bradykinin, 10  $\mu\text{g}/\text{ml}$ ; histamine, 100  $\mu\text{g}/\text{ml}$ ; and serotonin, 1  $\mu\text{g}/\text{ml}$ . They were further diluted if necessary before use. Volumes of 0.1 ml of each sample were injected intradermally with 27-gauge hypodermic needles into the abdominal skin of rabbits and the back of guinea pigs and rats which had received Evans blue with  $^{125}\text{RISA}$  intravenously. Unless otherwise stated, 30 min later, the animals were killed with sodium Nembutal (Upjohn Co., Kalamazoo, Michigan) and the extravasated dye and the radioactivity of the punched out skin were determined.

As an experimental model of inflammation, the following two types of inflammation were used. (i) The Arthus reaction: This was elicited in the abdominal skin of BSA-immunized rabbits according to methods previously described (1). Unless otherwise stated, 0.1 ml of antigen (2.5 mg of bovine serum albumin (BSA); Mann Research Lab., New York) was injected intradermally at 48, 24, 12, 6, 4.5, 3, 2, 1 hr, 30, 10, and 5 min before injecting Evans blue and  $^{125}\text{RISA}$ . (ii) Thermal injury was induced in the abdominal skin of rabbits at  $56^\circ \pm 0.25$  for 20 sec, by using the burning apparatus of Seivitt (2), slightly modified. Lesions were induced at 6, 5, 4, 2.5, 1 hr and at 20 and 5 min before injecting dye and  $^{125}\text{RISA}$ . Thirty min later, the animals were killed and the extravasated dye and radioactivity were measured as described above.

**Results. The relationship between extravasated dye and radioactivity of skin lesions.** First, the radioactivity of skin lesions of varying intensities was determined. Then following formamide extraction, the total amount of extravasated dye ( $\mu\text{g}$ ) in a particular skin lesion, was calculated by means of the standard calibration curve. As shown in Fig. 1, a linear response was obtained between about 1000 and 13,000 counts, corresponding to about 0–18  $\mu\text{g}$  of Evans blue.

**Recovery of Evans blue given intradermally.** 0.1 ml of Evans blue was injected intradermally at various concentrations. The skin

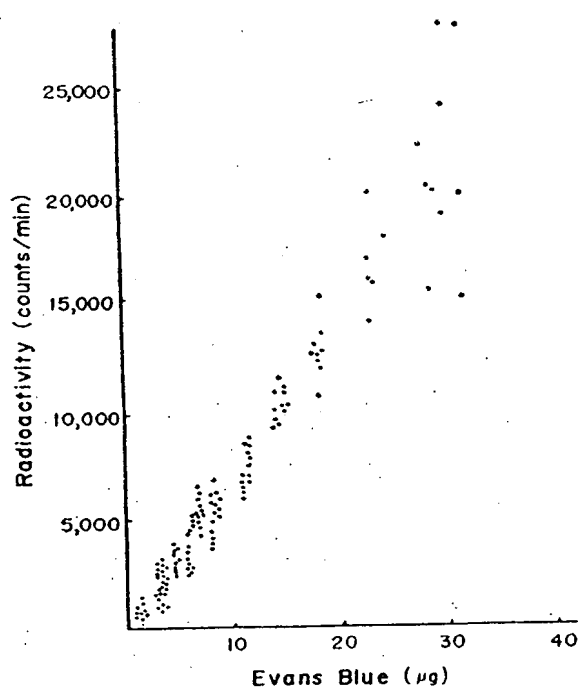


FIG. 1. The dots show radioactivity of skin lesions of varying intensity expressed as counts per minute per lesion. They also show the relationship between radioactivity of the individual skin lesions and the amount of Evans blue extracted from the same lesions.

was removed 30 min after injection. The dye was extracted and measured.

As illustrated in Table I, the recovery of dye was over 95% in all animals tested (rabbits, guinea pigs, and rats). This shows that the dye given interdermally can be recovered from the skin almost completely.

*Extraction of dye from skin sites treated with bradykinin, histamine, and serotonin.*

TABLE I. Recovery of Evans Blue Given Intradermally.

Dye injected ( $\mu\text{g}$ )	Dye recovered ( $\mu\text{g}$ ) <sup>a</sup>			Yield (av; %)
	Rabbits	Guinea pigs	Rats	
5.0	5.0	4.9	4.8	Over 96
10.0	10.1	9.8	9.8	Over 98
30.0	29.6	29.8	29.3	Over 97
50.0	48.8	49.2	48.1	Over 95
75.0	74.4	74.5	73.6	Over 98
100.0	98.2	97.6	—	Over 97

<sup>a</sup> Mean values of 5 experiments.

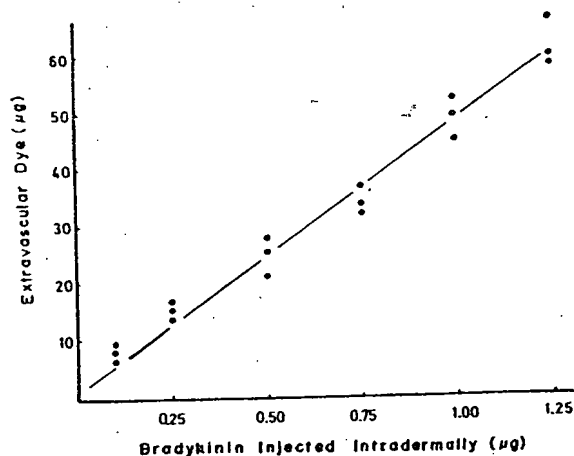


FIG. 2. Dose-response curve of bradykinin: volume injected was 0.1 ml; skin was removed 30 min after intradermal injections; Evans blue and  $^{125}\text{I}$ RISA were injected intravenously as described in the text.

0.1 ml of synthetic bradykinin was injected intradermally, in graded concentrations, into the abdominal skin of rabbits and the back of guinea pigs which had received Evans blue and  $^{125}\text{I}$ RISA intravenously. As shown in Fig. 2, the dose-response relationship shows a straight line between concentration of 0.10 and 1.25  $\mu\text{g}/\text{ml}$  of bradykinin.

Similar injections, using histamine and serotonin, were given to guinea pigs and rats, respectively. The same relationship between concentrations of 0.3 and 10  $\mu\text{g}/\text{ml}$  of histamine and 0.1 and 0.5  $\mu\text{g}/\text{ml}$  of serotonin

were obtained. These results indicate that this assay is useful for estimating increased vascular permeability in the skin induced with known chemical mediators.

*Enhanced vascular permeability in experimental models.* The time courses of vascular permeability in cutaneous Arthus reactions and moderate thermal injury in rabbits were tested. As shown in Fig. 3, the general pattern of vascular response appeared to be biphasic. The early response appeared in 5 min, lasted 20–30 min and decreased thereafter in both responses. The late response reached its maximum in 4–5 and 2 hr, respectively; and disappeared in 10–12 and 4–5 hr, respectively. This indicates that the assay allows accurate measurement of increased vascular permeability in cutaneous Arthus reaction and in thermal injury.

*Discussion.* The earliest attempts to estimate quantitatively the amounts of accumulated dye in inflamed skin sites were based on "mean lesion diameter" (3) or by comparing the color intensity with a series of color standards (4). However, evaluation of experimental results in such tests often lacks precision. Attempts to extract the dye from the skin were cumbersome in most instances. Beach and Steinetz (5) used acid digestion, Judah and Willoughby (6) pounded the skin frozen at  $-70^\circ$ , Carr and Wilhelm (7) homo-

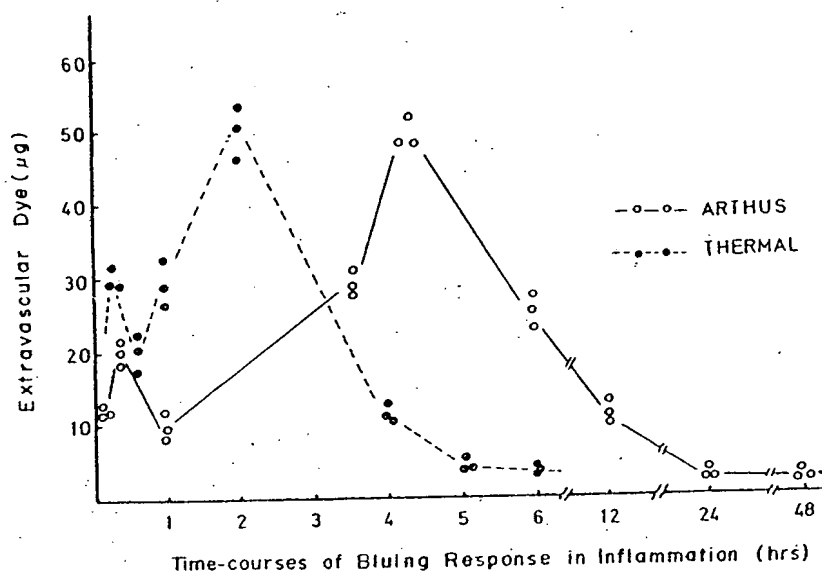


FIG. 3. Time-course of permeability changes in Arthus reaction and thermal injury.

genized the skin mechanically, whereas Nitta *et al.* (8). extracted pontamine sky blue from the skin in two steps: (a) denaturation and dehydration of tissues with dioxane, and (b) elimination of the tissue lipid with organic solvents such as methanol, ethanol, and ether.

In 1962, Frimmer and Müller (9) demonstrated that the extravasated dye could be extracted from the skin with formamide and estimated quantitatively by colorimetry. The extraction being performed at 80° for 24 hr induced a color change ranging from green to dark brown. When extracted under the conditions described in this report, no color changes were observed. Especially at 45°, the incubation time can be prolonged until the blue color of the skin completely disappears without any color changes taking place. Good results were obtained with this method in two studies in which enhanced vascular permeability has been measured (10, 11). In these studies large amounts of animals had to be used because of considerable variation in bluing from animal to animal obtained with intense bluing reactions. As shown in Fig. 1 not much scattering is obtained within a certain range. This means that the material to be tested has to be prepared in such a way as to give a bluing response not exceeding 20  $\mu$ g of Evans blue or about 13,000 cpm/lesion. If a certain standard (*e.g.*, synthetic bradykinin or histamine) which falls within the linear dose-response is used in each experiment, one can compare it visually with the bluing induced by the unknown permeability factor. If the latter gives too intense a reaction it can be further diluted. The assay with the <sup>125</sup>I-labeled serum albumin is simple, sensitive and rapid. It allows quantitation within minutes after completion of the experiment.

In addition to permeability tests with known chemical mediators (Fig. 2) it was shown that this assay is applicable to the time course study of enhanced vascular permeability in cutaneous Arthus reactions and thermal injury (Fig. 3). These results show that this assay permits quantitation of enhanced vascular permeability in studies dealing with certain immune reactions and of inflammatory lesions induced with various chemical mediators and of other phlogistic agents.

**Summary.** A simple physicochemical assay for the quantitation of enhanced vascular permeability in inflammation was described. It was shown that the assay is applicable to the study of inflammatory lesions induced with known chemical mediators, to the study of enhanced vessel permeability associated with the Arthus reaction, and that associated with thermal injury.

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SUPPRESSIVE EFFECT OF HUMAN BLOOD COAGULATION FACTOR XIII  
ON THE VASCULAR PERMEABILITY INDUCED BY ANTI-GUINEA PIG  
ENDOTHELIAL CELL ANTISERUM IN GUINEA PIGS

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Abstract

We investigated the effect of blood coagulation factor XIII (FXIII) on enhanced permeability induced by anti-endothelial cell antiserum, that was produced by the immunization of guinea pig endothelial cells with adjuvant into rabbits repeatedly. We have found that this antiserum reacts to human and guinea pig endothelial cells but not guinea pig fibroblast cells. The permeability was enhanced by intradermal injection of 400-fold dilution of this antiserum into dorsal skin of guinea pigs. The mixture of equal volume of antiserum and FXIII was intradermally injected into dorsal skin of guinea pig after Evans blue injection, and 15 minutes later the quantity of Evans blue at the each injection site was determined. We recognized the suppressive effect of FXIII on the dye leakage. We also studied the suppressive effect on swelling induced by the antiserum. After the subcutaneous injection of the mixture of antiserum and FXIII into the back of guinea pigs, we measured the thickness of skins at the injection site after day 1, 2 and 3. As a result, FXIII significantly suppressed the swelling. We found that FXIII suppresses the acute and subacute permeability enhancement. These results suggest that FXIII plays an important role on an inflammatory site and that it may exert as an anti-inflammatory protein.

Blood coagulation factor XIII (FXIII), the last enzyme in the

Key words: Factor XIII, anti-endothelial cell antiserum, vascular permeability, anti-inflammatory protein, Schönlein Henoch purpura



blood coagulation cascade, is a transamidase that catalyzes the formation of  $\gamma$  - glutamyl -  $\epsilon$  - lysyl peptide crosslinks between polypeptide chains in adjacent fibrin monomers and other plasma proteins(1,2,3). Crosslinks of each fibrin molecule caused a marked increase in the rigidity of the clot network(4). On the other hands, the crosslinks between fibrin and cellular matrix protein such as fibronectin may exert to connect fibrin molecules with the injury sites(5). It is well known that clots play an important role in the prevention of further tissue damage and in subsequent wound healing(6). Schönlein Henoch purpura (SHP) is characterized by hemorrhagic skin lesions, abdominal symptoms including gastro-intestinal bleeding, renal involvement with proteinuria and hematuria and swelling of joints(7). The symptoms are ascribed to generalized inflammation of arterioles and capillaries. That is, the local changes of the coagulation and fibrinolytic system due to immunoreaction were induced in the affected vessels. In 1977, Henriksson and colleagues described a lowering of FXIII activity during the acute phase of this disease(8). The mechanism of the decrease of FXIII activity in the acute phase of SHP has not yet been clarified. Destruction of FXIII molecules by protease derived from leukocytes which migrated into the inflammation sites has been proposed(9). In this connection, Kamitsuji and Fukui et al. reported that the administration of FXIII concentrate may contribute to the improvement of gastro-intestinal complications of SHP patients(10). Recently FXIII concentrate (Fibrogammin P) is used for the treatment of SHP patients(11). According to Matsuoka(12), Bowie et al.(13) and Ito et al.(14), this vasculitis of SHP is regarded as the immunovascular disease that antibody-antigen complexes on the vascular capillary endothelial cells enhances the vascular permeability. Consequently non-thrombocytopenic purpura caused by the injection of anti-endothelial cell antiserum(15). In the present study, we investigated whether or not human FXIII suppresses the enhancement of permeability and swelling induced by anti-endothelial cell antiserum in guinea pigs.

#### MATERIALS AND METHODS

##### Materials

Materials were purchased from the following suppliers: Dulbecco phosphate buffer, Dulbecco MEM, FCS (Gibco, USA), ECGS (Calbiochem, USA), Freund's adjuvant (Difco, USA), FITC conjugated anti-rabbit IgG (Cappel, USA), Evans blue, potassium hydroxide (KOH, Kanto Kagaku, Japan), phosphoric acid (Wako Pure Chemical, Japan), Guinea pig complement (Kyokuto, Japan), and Human FXIII (Fibrogammin P, Behringwerke, FRG).

*Preparation of anti-guinea pig endothelial cell antiserum*

Guinea pig endothelial cells were isolated from the main artery and vena cava(16), then cells were inoculated into tissue culture dishes and incubated for several days with Dulbecco MEM containing 15% FCS and 37.5 µg/ml ECGS till reaching confluency. Confluent monolayer was harvested by a cell scraper. The cells were rinsed twice with Dulbecco phosphate buffered saline(pH 7.2). These cells were used as an antigen for the production of anti-endothelial cell antiserum. The antiserum was obtained from rabbits immunized with emulsion of Freund's complete adjuvant with guinea pig endothelial cells, and boosted with emulsion of Freund's incomplete adjuvant. After several times of boosting, the antibody titer was measured with guinea pig endothelial cells by the methods of cytolysis and indirect immunofluorescence microscopy using FITC conjugated anti-rabbit IgG(17).

*Measurement of antibody titer of anti-endothelial cell antiserum*

Confluent monolayer of guinea pig endothelial cells in a 96-well plate was incubated with 50 µl of variously diluted antiserum in Dulbecco MEM-15% FCS for 30 min. The medium was then replaced to 50 µl of 5% guinea pig complement in Dulbecco MEM-15% FCS and the cells were further incubated for 30 min. After addition of 10 µl of trypan blue solution, the cell layers were photographed to evaluate the extent of cell lysis. Indirect immunofluorescence microscopy was done as follows. The antiserum was serially diluted two times. The diluted antiserum was then incubated with the main artery at room temperature for 1 hour and rinsed 3 times with Dulbecco phosphate buffer. After washing, 1000-fold dilution of FITC conjugated anti-rabbit IgG was added to the sections, incubated for 30 minutes at room temperature, and washed 3 times with Dulbecco phosphate buffer. All sections were observed by a Nikon microscope equipped with a mercury lamp. The titer was taken as a highest dilution which gave a fluorescent staining just above the background staining of normal serum controls.

*Duration of activity of permeability enhancement*

Measurement of permeability was studied according to Yamamoto et al.(18). A 100 µl portion of 50-fold diluted antiserum was intradermally injected into the back of a guinea pig before intravenous injection of 0.5 ml of 1 % Evans blue. After 15 minutes of the Evans blue injection, the back skins were harvested and the blue lesions were observed.

*Suppressive effect of FXIII on the permeability enhancement*

A 100 µl portion of either each diluted antiserum or the mixture of equal volume of FXIII and the diluted antiserum was

intradermally injected into the dorsal skin of guinea pigs after intravenous injection of Evans blue. After 15 minutes, skins were harvested and blue lesions in the skins were observed.

*Extraction of Evans blue from guinea pig skins*

Evans blue was extracted from skins, soaked with 1 ml of 1 M KOH solution, and incubated at 37°C overnight. After the incubation, 3 ml of 0.6 N phosphoric acid and 3 ml of FRIGEN (Behringwerke, FRG), a defatting agent, was added to each tube and mixed for 30 sec. with a Vortex mixer. Each tube was centrifuged at 3000 rpm for 15 minutes, and the absorbance of the supernatant was measured at 620 nm(19).

*Suppressive effect of FXIII on the swelling*

One milliliter of equal volume mixture of FXIII and the intact antiserum was subcutaneously injected into the dorsum of guinea pigs. After days 1, 2 and 3, the skins were harvested and the thickness was measured with a slide caliper at injection sites as a marker of swelling. The swelling was shown by the difference of the thicknesses between a injection and a non-injection site.

## RESULTS

*Characterization of polyclonal anti-guinea pig endothelial cell antiserum*

The antibody titer was determined with guinea pig endothelial cells by the methods of cytolysis and indirect immunofluorescence microscopy using FITC conjugated anti-rabbit IgG. As a result, the 50% cytolysis was observed by the 60-fold dilution of antiserum, and the fluorescence was observed by 400-fold dilution. The antiserum exhibited the reactivity with not only guinea pig but also human endothelial cells. However it did not react with guinea pig fibroblasts. When the cryosection of the main artery of a guinea pig was used for the indirect immunofluorescence test, the fluorescence was observed on the inner membrane which was seemed to be endothelial cell. It was also found that the antiserum reacted with the extracellular matrix proteins produced by endothelial cells(data not shown).

*Enhanced permeability*

First, we studied whether this antiserum induced the permeability in guinea pigs. The variation of permeability after intradermal injection is shown in Fig. 1. The permeability reached the maximum within 5 minutes. This activity for enhancing the permeability almost disappeared within 30 minutes after the injection. This permeability enhancing phenomenon was classified as a short lasting reaction. We next investigated the dose response of this antiserum. As shown in Fig. 2, the activity of

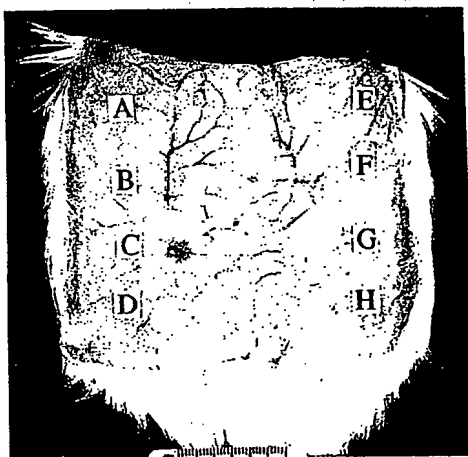


FIG. 1.

Time course of permeability enhancement induced by anti-endothelial cell antiserum. Antiserum was injected into a guinea pig at varying times before intravenous dye injection. Time 0 means an intradermal injection immediately after intravenous dye injection. (A): antiserum, 60 min, (B): antiserum, 30 min, (C): antiserum, 0 min, (D): saline, 30 min, (E): rabbit serum, 60 min, (F): rabbit serum, 30 min, (G): rabbit serum, 0 min, (H): saline, 0 min

enhancing the permeability is recognized by 400-fold dilution of antiserum. The effect of FXIII was examined on the vascular permeability induced by the antiserum. In this experiment, the mixture of antiserum was injected with various concentration of FXIII. As shown in Fig. 3, FXIII shows the suppressive effect on the dye leakage in a dose dependent fashion. We obtained a result that both 200-fold and 400-fold diluted antiserum exhibit the same tendency. Thus the effect of FXIII was examined in 10 guinea pigs and the dye leakage was measured in extravascular space. As shown in Fig. 4, FXIII exhibited the suppressive effect in a dose dependent manner.

#### *Suppressive effect of FXIII on the swelling*

When the antiserum was subcutaneously injected into a dorsal skin of guinea pig, edema, in addition to hemorrhage was observed at injection site(20). Thus we examined the suppressive effect of FXIII on the swelling. On injecting the mixture of FXIII and antiserum, the edema was significantly suppressed by FXIII on day 1 and 2(Fig. 5). This result indicates that FXIII suppresses the permeability in the acute and the subacute phase as well.

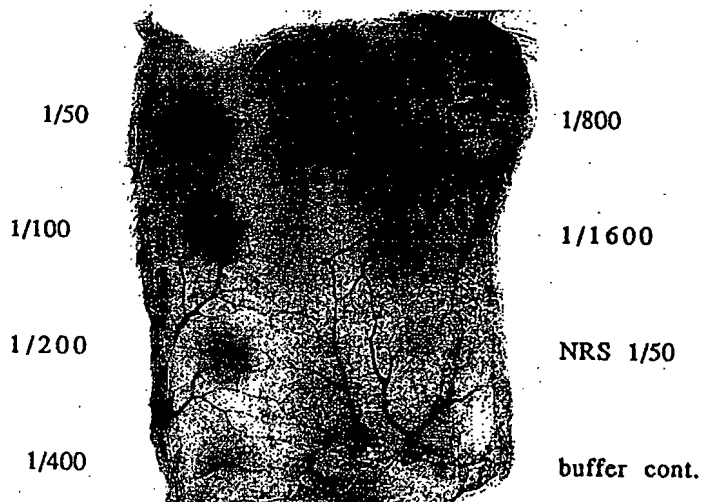


FIG. 2.

Dose response of anti-endothelial cell antiserum in a guinea pig. Each sample was injected immediately after a intravenous dye leakage. NRS: Normal rabbit serum

#### DISCUSSION

For more than 20 years after its detection of FXIII, many authors have reported that a clotting factor, FXIII, influenced a lot of other systems and thus it was often termed a connective tissue factors (21). The fibrin stabilizing effect is an example of general properties of this factor which crosslinks proteins with suitably configured  $\epsilon$ -lysyl- and  $\gamma$ -glutamyl-residues. Many kinds of proteins are listed as substrates for FXIII, e.g. fibrin(1), collagen(22), fibronectin(5), actin(23) and factor V(24). In this context, the binding of biogenic amines to proteins by FXIII may also participate in the elimination of toxic substances like histamine. FXIII concentrate has been recently used not only for the promotion of the wound healing but also for the treatment of Schönlein Henoch Purpura(SHP) (6,10). The clinical effects of FXIII on SHP are probably due to the stabilization of microvasculature leading to a reduction of the leakage at inflammatory sites. Pilger et al(25) has reported that FXIII shows the suppressive/sealing effect in a scleroderma patient. However none of these reports showed the sealing/suppressive effect on the permeability by FXIII in animal studies. This vasculitis of SHP is regarded as the immunovascular disease that

the vascular permeability is enhanced by the formation of the antigen-antibody complex not with standing ambiguity of trigger which may include drugs, foods, insect bites or bacterial infections(11,12,13,14). Thus we tried to demonstrate the suppressive effect of FXIII on permeability enhancement induced by anti-endothelial cell antiserum. As shown in Figs. 1 and 2, anti-endothelial cell antiserum induces the enhancement of permeability. This phenomenon can be caused by factors such as complement fragments and histamine etc. which are produced by the activation of complement system after complex formation of antiserum with endothelial cells(11,12,13,14). As this phenomenon shows the dose dependent manner by antiserum, condition of SHP patients may be influenced seriously depending on the extent of the antibody generation. SHP patients show the increase of plasma level of IgA and the imbalance of serum IgG subclass and IgM(13,14,26). As shown in Figs. 3, 4 and 5, FXIII suppresses the vascular permeability in acute phase and the edema in subacute phase. These results are supported by some clinical studies. Kamitsuji et al.(10) and Fukui et al.(11) have reported that FXIII shows the suppressive effect on the swelling of joints of SHP patients.

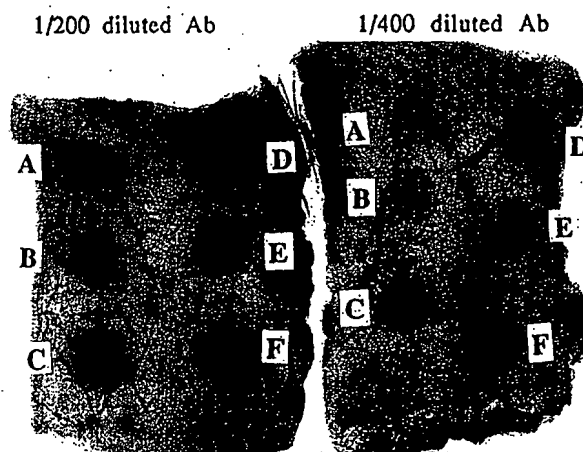


FIG. 3.

Suppressive effect of FXIII on the permeability enhancement induced by anti-endothelial cell antiserum. FXIII was used with the final concentration at a injection site of (A), 3.0 U; (B), 1.5 U; (C), 0.75 U; (D), 0.38 U; (E), medium control. The mixture of FXIII and either 200- or 400- fold diluted antiserum was injected immediately after the intravenous injection of dye.

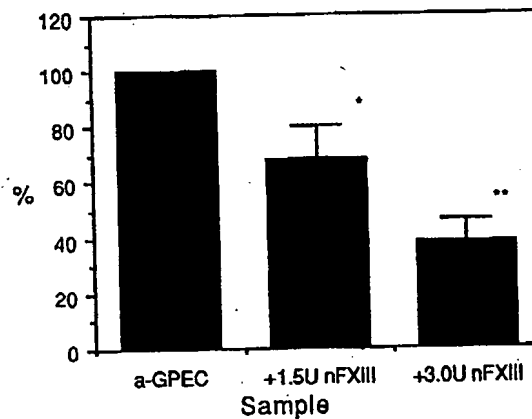


FIG. 4

Suppressive effect of FXIII on the permeability induced by anti-guinea pig endothelial cell antiserum. Extraction of Evans blue at the injection site was according to the materials and methods.  $n=10$ , \*:  $p<0.05$ , \*\*:  $p<0.01$ . In this experiment, we used the 300-fold diluted anti-endothelial cell antiserum as a permeability inducer. FXIII was mixed with antiserum, then the mixture was injected intradermally.

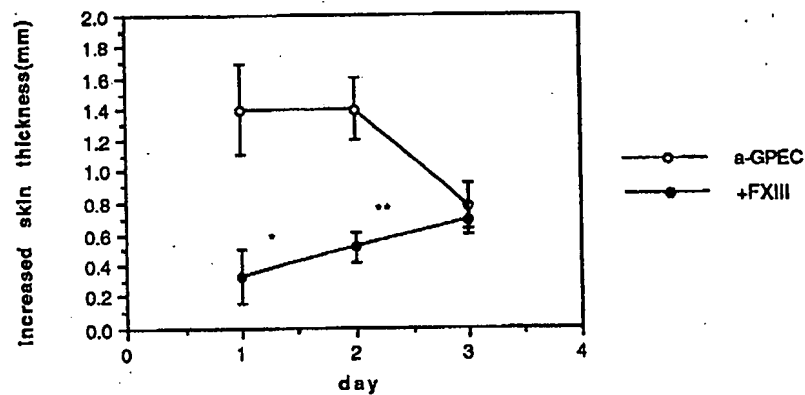


FIG. 5.

Effect of FXIII on the swelling induced by anti-guinea pig endothelial cell antiserum. Open circle (o) denotes the antiserum alone. Closed circle (●) denotes the FXIII plus antiserum.  $n=5$ , \*:  $p<0.05$ , \*\*:  $p<0.01$ .

Pilger et al. (25) reported that FXIII also shows the suppressive effect on vascular permeability in scleroderma patients. These results suggest that FXIII may crosslink cellular matrices to prevent the opening of the space between cells (27) and that it may crosslink the enhancing factors for the permeability (21). We have succeeded in demonstrating the suppressive effect of FXIII on vascular permeability in an animal study. This study indicates that FXIII may play a crucial role in an inflammatory site. Consequently it seems that FXIII therapies are necessary for the treatment of some inflammatory diseases (28,29).

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## Tumor Cells Secrete a Vascular Permeability Factor That Promotes Accumulation of Ascites Fluid

**Abstract.** Tumor ascites fluids from guinea pigs, hamsters, and mice contain activity that rapidly increases microvascular permeability. Similiar activity is also secreted by these tumor cells and a variety of other tumor cell lines in vitro. The permeability-increasing activity purified from either the culture medium or ascites fluid of one tumor, the guinea pig line 10 hepatocarcinoma, is a 34,000- to 42,000-dalton protein distinct from other known permeability factors.

Abnormal accumulation of fluid commonly accompanies solid and particularly ascites tumor growth (1). To investigate the mechanism of tumor ascites formation, we measured the rates of influx and efflux of  $^{125}\text{I}$ -labeled human serum albumin (HSA) at various times after the implantation of tumor cells in the peritoneal cavities of guinea pigs. We detected a markedly increased influx of HSA as early as 1 hour after intraperitoneal injection of guinea pig line 10 hepatocarcinoma cells, which provoke a substantial accumulation of ascites fluid (Table 1). In contrast, efflux of HSA from the peritoneal cavities of animals bearing line 10 tumors did not change significantly, even with progressive tumor growth (2).

To establish whether the increased influx of fluid induced by tumor cells reflects an alteration in vessel permeability, we injected animals intravenously with colloidal carbon. Examination of the peritoneal cavities of strain 2 guinea pigs, Syrian hamsters, and A/Jax mice bearing syngeneic ascites tumors (line 10, HSV-NIL8, and TA3-St, respectively) revealed that many venules of the peritoneal wall, diaphragm, mesentery, and gastrointestinal serosal surfaces were heavily labeled with colloidal carbon, indicating increased permeability; comparable vessels in control animals were not labeled.

These observations suggest that tumor ascites may be attributable to alterations in the permeability of vessels lining the peritoneum. To investigate the basis for this increased permeability, we used the Miles assay (3) to test ascites fluid for the presence of factors that increase vascular permeability (Table 2 and Fig. 1).

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Ascites fluid from line 10 guinea pig and TA3-St mouse carcinomas and the HSV-NIL8 hamster sarcoma all markedly increased local cutaneous vascular permeability. The increase was evident after 1 minute and maximal within 5 to 10 minutes. By contrast, platelet-poor plasma samples from the same species (Table 2 and Fig. 1) and oil-induced peritoneal exudate fluids (4) had little or no activity. The tumor ascites permeability-increasing activity was not inhibited by soybean trypsin inhibitor (1000  $\mu\text{g}/\text{ml}$ ); therefore, it is not PF/dil (5), a permeability factor unmasked when serum is diluted  $\geq 1:100$  (6).

We previously reported that line 10

Table 1. Peritoneal vessel permeability. Guinea pigs (400 g) were injected intraperitoneally with  $3 \times 10^7$  line 1 or line 10 tumor cells (17) or with peritoneal exudate cells in Hanks balanced salt solution (HBSS) and immediately thereafter were injected intravenously with  $^{125}\text{I}$ -labeled HSA ( $5 \times 10^6$  dis/min). One hour later the animals were exsanguinated under ether anesthesia, and peritoneal fluid was collected following intraperitoneal injection of 20 ml of heparinized (10 U/ml) HBSS. For each animal total radioactivity in the ascites fluid was determined and normalized for that in the blood; influx of HSA was computed as the ratio of total disintegrations per minute in peritoneal fluid to those per milliliter of blood. Net influx was determined by subtracting influx values for control animals. Values are means  $\pm$  standard errors ( $N = 4$ ).

Type of cells injected intraperitoneally	Net peritoneal influx of HSA
Line 1	$0.09 \pm 0.04$
Line 10	$0.41 \pm 0.08$
Line 10 + immune IgG (2 mg)	$0.11 \pm 0.03$
Peritoneal exudate	0

tumor cells release a vascular permeability-increasing activity in serum-free culture (7). This activity is not inhibited by soybean trypsin inhibitor (200  $\mu\text{g}/\text{ml}$ ), and its production by cells in vitro requires protein synthesis (complete inhibition by 20  $\mu\text{g}$  of cycloheximide per milliliter). Many other tumor cell lines also release permeability-increasing activity in serum-free culture, including guinea pig 104 C1 fibrosarcoma, hamster HSV-NIL8 sarcoma, rat sarcomas B77 Rat 1 and RR 1022, and mouse TA3-St carcinoma, MOPC 21 myeloma, and polyoma BALB/c 3T3 sarcoma. Line 1 guinea pig hepatocarcinoma cells release one-fourth the activity released by line 10 cells, a finding that may explain the relative ability of these cells to promote HSA influx (Table 1) and ascites fluid accumulation (the volume of line 1 ascites fluid was routinely one-fourth that of line 10). Oil-induced guinea pig peritoneal exudate cells ( $> 70$  percent macrophages) neither increase the influx of HSA into the peritoneum (Table 1) nor secrete detectable permeability-increasing activity in vitro. Guinea pig fibroblasts and smooth muscle cells release approximately one-eighth the activity released by comparable numbers of line 10 cells (8).

We next purified both the ascites and tissue culture permeability factors from a single tumor, the line 10 guinea pig carcinoma. Permeability-increasing activities from both sources chromatographed identically as single peaks on columns containing Sephadex G-150, heparin-Sepharose, or hydroxylapatite (9) and electrophoresed as a single peak with an apparent molecular weight of 34,000 to 42,000 on sodium dodecyl sulfate-polyacrylamide gels (Fig. 2). Using the heparin-Sepharose, hydroxylapatite, and electrophoretic steps in tandem, we purified the permeability-increasing activity approximately 1200-fold from serum-free conditioned medium and approximately 10,000-fold from ascites fluid. As little as 200 ng ( $5 \times 10^{-12}$  mole) of the purified material increased vascular permeability to a degree equivalent to that induced by 1.25  $\mu\text{g}$  ( $4 \times 10^{-9}$  mole) of histamine.

Rabbits immunized with the purified permeability factor secreted by line 10 cells in vitro produced an immunoglobulin G (IgG) that bound and neutralized virtually all the permeability-increasing activity in undiluted line 10 and line 1 tumor ascites fluids (Table 2) and in line 10 and line 1 culture media. This antibody also blocked the peritoneal influx that follows intraperitoneal injection of line 10 tumor cells (Table 1). In every

Table 2. Vascular permeability as determined by the Miles assay. Guinea pigs were injected intravenously with  $^{125}\text{I}$ -labeled HSA ( $1.5 \times 10^6$  dis/min) in 1 ml of saline containing 0.5 percent Evans Blue dye. Samples to be tested for permeability-increasing activity, in isotonic solution and at neutral pH, were injected intradermally in a volume of 0.1 ml. After 20 minutes the animals were exsanguinated under ether anesthesia. Test sites were excised and quantitated for  $^{125}\text{I}$  in a gamma counter. The number of net disintegrations per minute extravasated was determined by subtracting values for control sites injected with saline. Each animal also received a series of intradermal histamine injections; these sites served as reference points for the calculation of histamine equivalents. B.L., below limit of quantitation (0.6  $\mu\text{g}$  histamine).

Substance injected intradermally	Net disintegrations per minute $^{125}\text{I}$ -HSA extravasated (mean $\pm$ standard error) ( $N = 3$ to 7)	Histamine equivalent* ( $\mu\text{g}$ )
Hamster plasma	$70 \pm 176$	B.L.
Hamster ascites (HSV-NIL8)	$15,309 \pm 1,508$	1.3
Guinea pig plasma	$1,989 \pm 1,070$	B.L.
Line 1 ascites	$69,609 \pm 6,850$	5.5
Line 1 ascites + preimmune IgG (80 $\mu\text{g}$ )	$70,321 \pm 2,567$	5.5
Line 1 ascites + immune IgG (80 $\mu\text{g}$ )	$3,935 \pm 1,568$	B.L.
Line 10 ascites	$92,472 \pm 4,886$	10.0
Line 10 ascites + preimmune IgG (80 $\mu\text{g}$ )	$93,756 \pm 1,171$	10.0
Line 10 ascites + immune IgG (80 $\mu\text{g}$ )	$7,187 \pm 930$	B.L.
Line 10 serum-free culture supernatant†		
After 1 hour of culture	$1,054 \pm 60$	B.L.
After 5 hours of culture	$3,610 \pm 295$	0.7
After 24 hours of culture	$21,565 \pm 617$	2.5

\*A plot of net disintegrations per minute extravasated in response to histamine versus the logarithm of the number of micrograms of histamine injected generated a straight line (histamine range, 0.6 to 10  $\mu\text{g}$ ).  
†Derived from cultures containing  $2.5 \times 10^6$  cells per milliliter.

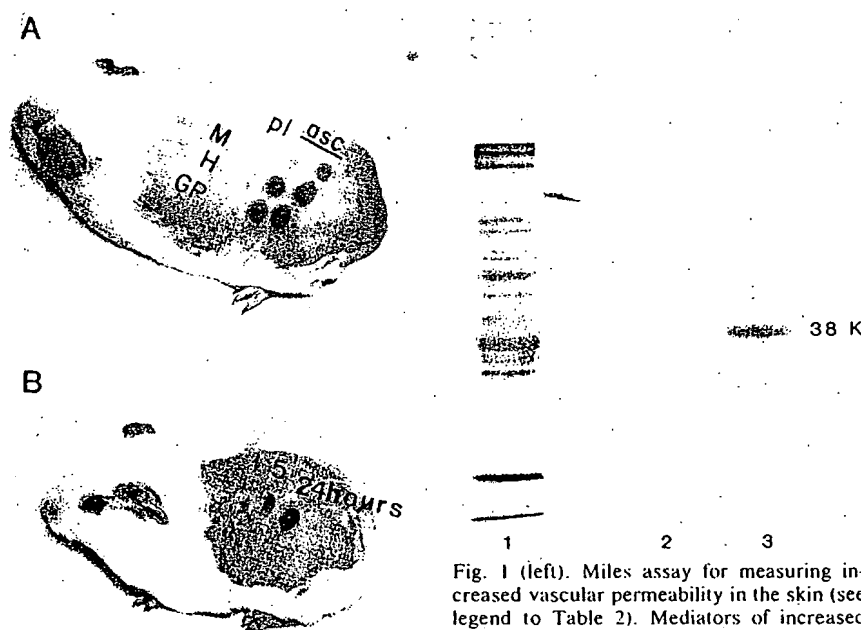


Fig. 1 (left). Miles assay for measuring increased vascular permeability in the skin (see legend to Table 2). Mediators of increased permeability cause bluing at the site of intradermal injection within 5 minutes, whereas control substances such as saline elicit no

response. Abbreviations in (A): *pl*, control plasma; *asc*, ascites fluid; *M*, mouse TA3-S1 tumor; *H*, hamster HSV-NIL8 tumor; and *GP*, guinea pig line 10 tumor. In (B), line 10 cells were cultured ( $1 \times 10^6$  cell/ml) in serum-free Dulbecco's modified Eagle's medium and conditioned media was harvested at 1, 5, and 24 hours as indicated. Fig. 2 (right). Resolution of the permeability-increasing activity on sodium dodecyl sulfate-polyacrylamide slab gels (16). Samples were electrophoresed without reduction at  $4^\circ\text{C}$  in a 7.5 percent polyacrylamide gel containing 0.1 percent sodium dodecyl sulfate and washed for 1 hour at  $4^\circ\text{C}$  in 2.5 percent Triton X-100 and then in phosphate-buffered saline for 1 hour. The gel was sliced, individual slices were extracted, and dialyzed extracts were tested for activity by the Miles assay. Track 1 shows the stained pattern of concentrated line 10 serum-free culture medium. All the activity in track 1 (total recovery was regularly 50 percent) was confined to two adjacent slices (reelectrophoresed in tracks 2 and 3) composing the 34,000 to 42,000 molecular weight region. Line 10 ascites fluid permeability-increasing activity was found to electrophorese identically (molecular weight 34,000 to 42,000) with the activity in line 10 culture medium.

ease. The permeability-increasing activity of the immunization (preimmune IgG) was without effect. The IgG from immunized animals (immune IgG) also neutralized the permeability-increasing activity released in culture by an unrelated tumor, the 104 C1 guinea pig fibrosarcoma (10), but not the activity of guinea pig PF/dil or the low levels of activity released by guinea pig fibroblasts or smooth muscle cells.

As determined by light and electron microscopy, line 10 permeability factor did not cause endothelial cell damage or mast cell degranulation. Vessels responded equally well to multiple challenges with equivalent doses of line 10 permeability factor administered 30 minutes apart; the effect of a single intradermal injection was rapid (evident within 5 minutes) and transient (little residual increased permeability was detectable 20 minutes after injection), providing further evidence that line 10 permeability factor is not toxic to blood vessels. It does not resemble bradykinin (molecular weight, 1200), plasma kallikrein (108,000), or leukokinins (2500). Leukokinins (11) are generated in ascites fluids under nonphysiological conditions (pH 3.8 and  $37^\circ\text{C}$  for 24 hours) by a mechanism sensitive to 1  $\mu\text{M}$  pepstatin A. Line 10 permeability factor is present in fresh, unmanipulated ascites fluid (pH 6.4 to 6.9), and its action is unaffected by 20  $\mu\text{M}$  pepstatin A. Lymphocyte permeability factors with molecular weights of 12,000 (12) and 39,000 (13) have been reported; however, unlike line 10 permeability factor, the latter increases vascular permeability only after a latent period of 20 minutes. The effects of line 10 permeability factor are not mediated by histamine. Guinea pigs treated with the antihistamines mepyramine (5  $\mu\text{mole/kg}$  subcutaneously) plus cimetidine (500  $\mu\text{mole/kg}$ ) (14) responded normally to line 10 permeability factor, although the action of 20  $\mu\text{g}$  of histamine was blocked. It is also unlikely that the effects of this factor are mediated through prostaglandin synthesis. Neither systemic (14  $\mu\text{mole/kg}$ , intraperitoneally) nor local (2 nmole, intradermally) treatment with indomethacin (15) affected the response of vessels to the permeability factor.

In conclusion, vessels lining the peritoneal cavities of guinea pigs, hamsters and mice bearing ascites tumors display markedly greater permeability than do the same vessels in control animals. This increased permeability is apparently due to the presence in ascites fluid of a potent permeability factor not found in normal plasma or serum. The permeability factors found in guinea pig line 10

culture medium or ascites fluids appear to be identical. In addition, they are antigenically related to permeability factors produced by guinea pig line 1 or 104 C1 tumor cells. Secretion of permeability-increasing activity appears to be a common feature of tumor cells, and may contribute to the abnormal accumulation of fluid associated with neoplastic disease.

**Note added in proof:** The immune IgG raised against line 10 permeability factor also neutralizes the rat dermal vessel permeability-increasing activity released by Walker rat carcinoma cells in culture. Preimmune IgG has no neutralizing effect.

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2. Clearance of labeled HSA from the peritoneal cavity was not impaired in tumor-bearing animals at any interval. For example, 7 days after intraperitoneal injection of  $3 \times 10^5$  line 10 tumor cells,  $58.4 \pm 4.2$  percent (mean  $\pm$  standard error) of the HSA (90 to 95 percent precipitable with trichloroacetic acid) remained in the peritoneal cavities after 2 hours;  $56 \pm 1.15$  percent remained in the controls.
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avoid complicating our analysis of diluted ascites fluid fractions with unmasked PF/dil (7), we added soybean trypsin inhibitor (20  $\mu$ g/ml) to all ascites fluid column fractions before assay.

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18. We thank B. Wildi, J. Feder, and R. D. Rosenberg for help and advice, J. Osage for technical assistance, and B. Zbar, R. Hynes, R. Garcia, T. Isomura, and J. Codington for cells. Supported by a grant from the Monsanto Company.

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## Yolk Pigments of the Mexican Leaf Frog

**Abstract.** Eggs of the Mexican leaf frog contain blue and yellow pigments identified as biliverdin and lutein, respectively. Both pigments are bound to proteins that occur in crystalline form in the yolk platelet. The major blue pigment is biliverdin IX  $\alpha$ . The eggs vary in color from brilliant blue to pale yellow-green depending on the amount of each pigment. These pigments may provide protective coloration to the eggs.

While studying the lipid composition of the eggs and embryos of the Mexican leaf frog, *Agalychnis dacnicolor* (1), we observed that their green coloration was due to the presence of two pigments, one blue and one yellow, which together produce blue, blue-green, or yellow-green eggs. We have now identified the major blue pigment as biliverdin IX  $\alpha$  and the major yellow pigment as lutein. The presence of the latter pigment is not surprising since lutein is widely distributed among plants and animals (2). Biliverdin occurs less often as a pigment, although it has been found in the dog placenta, in the shells of bird eggs, in the skin of fishes and amphibians (2, 3), in the eggs and larvae of the tobacco hornworm (4), and in the serum and eggs of *Xenopus* (5). It seems likely that the utilization of these two pigments by *A. dacnicolor* evolved as a mechanism for producing green eggs. The green coloration of leaf frog eggs, which are laid on green vegetation, may afford camouflage to protect eggs and embryos from predation. However, as far as we can ascertain, there have been no definitive studies on the adaptive value of green eggs, although the ecological implications deriving from a two-pigment system for egg coloration are apparent.

Six different batches of *A. dacnicolor* eggs (100 to 250 eggs) varying in color from brilliant blue to yellow-green were extracted with a 1:1 mixture of chloroform and methanol and a mixture of acetone and hydrochloric acid to obtain the yellow and blue pigments. The pigments were separated by column chromatography on silicic acid. Chloroform eluted the yellow pigment, and acetone

eluted the blue pigment. The pigments were further purified by preparative thin-layer chromatography (TLC). The yellow and blue pigments were localized in lipid-rich yolk platelets.

Yolk platelets, which were pale blue-green or pale yellow, were obtained by collagenase treatment of homogenized eggs, followed by differential centrifugation. Analysis by light microscopy of the isolated fresh yolk platelets revealed rounded rectangular platelets of different sizes, and electron microscopy showed that the platelets consisted of closely stacked crystalline arrays about 70 Å thick.

The ultraviolet to visible spectra of the silicic acid column-purified pigments from different eggs are given in Fig. 1, a and b. The blue pigment has major bands at 372 to 376 nm and 640 to 690 nm. The yellow pigments have major bands at 442 to 444 nm and 470 to 471 nm. The relative amount of the yellow and blue pigments in the various eggs was determined by the ratio of the absorbance at 442 nm to that at 650 nm. This ratio was correlated with the color of the egg: The ratio of brilliant blue eggs was 1.15, that of blue eggs was 1.7 to 2.3, that of green eggs was 3.4 to 3.6, and that of yellow-green eggs was 10.4.

The blue pigment has properties consistent with a biliverdin. Both the blue pigment and biliverdin (Sigma) were converted to methyl esters by treatment with methanolic HCl (Supelco). The dimethyl esters were purified by preparative TLC (Merck-Darmstadt silica gel 60 plates) using chloroform and methanol 9:1. Both had identical relative mobility ( $R_f$ ) values of 0.62, and gave a purple

## Selective Requirement for Src Kinases during VEGF-Induced Angiogenesis and Vascular Permeability

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### Summary

Src kinase activity was found to protect endothelial cells from apoptosis during vascular endothelial growth factor (VEGF)-, but not basic fibroblast growth factor (bFGF)-, mediated angiogenesis in chick embryos and mice. In fact, retroviral targeting of kinase-deleted Src to tumor-associated blood vessels suppressed angiogenesis and the growth of a VEGF-producing tumor. Although mice lacking individual Src family kinases (SFKs) showed normal angiogenesis, mice deficient in *pp60<sup>src</sup>* or *pp62<sup>yes</sup>* showed no VEGF-induced vascular permeability (VP), yet *fyn<sup>-/-</sup>* mice displayed normal VP. In contrast, inflammation-mediated VP appeared normal in Src-deficient mice. Therefore, VEGF-, but not bFGF-, mediated angiogenesis requires SFK activity in general, whereas the VP activity of VEGF specifically depends on the SFKs, Src, or Yes.

### Introduction

SFKs are important signaling molecules that respond to a wide range of stimuli including growth factors (Twamley-Stein et al., 1993; Broome and Hunter, 1996) and adhesion proteins in the extracellular matrix (Kaplan et al., 1994; Schwartz et al., 1995; Thomas and Brugge, 1997; Klinghoffer et al., 1999). Once activated, SFKs affect a wide range of downstream signaling events including the activation of MAP kinases (Courtneidge et al., 1993). While in vitro studies have elucidated a role for Src in cellular function, due to mechanisms of redundancies and compensation, mice lacking a single SFK (Soriano et al., 1991; Stein et al., 1994) have provided limited insight into the biological function of this important family of nonreceptor tyrosine kinases.

Previous studies have implicated SFKs in vascular cell proliferation. For example, v-Src, an oncogenic variant of Src, is known to promote hemangioma formation in chicks (Stoker et al., 1990), suggesting that under normal circumstances, c-Src or other SFKs may regulate the growth of blood vessels. To initially address this issue, we used avian- or murine-targeted retroviral delivery systems to express mutationally active or inactive

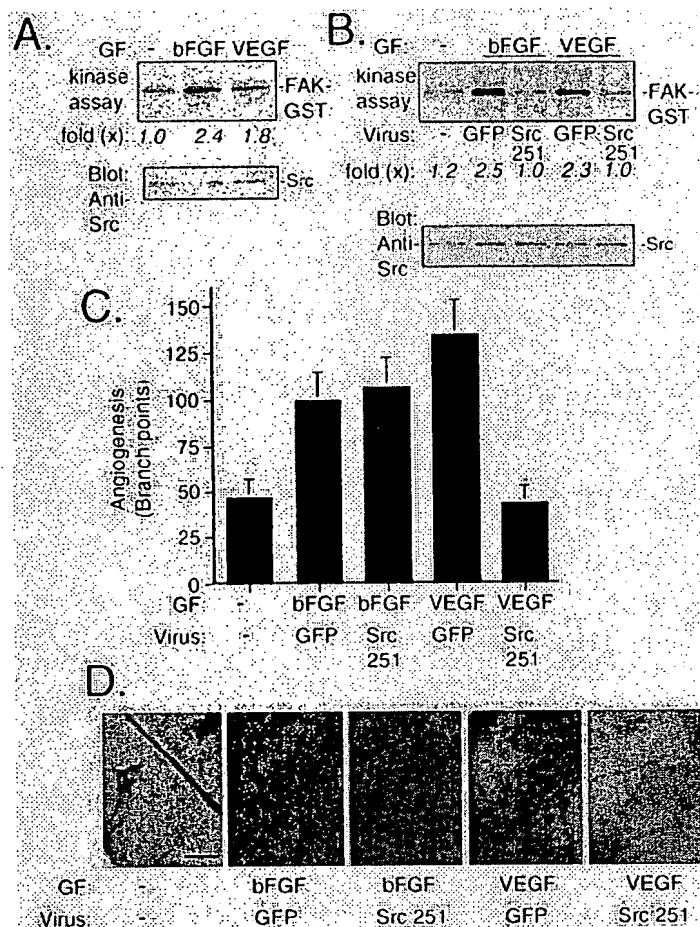
forms of Src or intact C-terminal Src kinase (Csk) to disrupt endogenous Src activity within the chick chorio-allantoic membrane (CAM) or mouse skin to directly evaluate the general role of Src kinases during angiogenesis. Evidence is provided here that Src kinase is required for VEGF-, but not bFGF-, mediated angiogenesis in both the chick embryo and the mouse. In fact, Src kinase activity was found to be required for endothelial cell survival during VEGF-mediated angiogenesis. While VEGF is an endothelial cell mitogen (Ferrara and Davis-Smyth, 1997), it was originally described for its vascular permeability (VP) activity (Senger et al., 1983; Connolly et al., 1989). In fact, VEGF is unique in this regard, as other growth factors such as bFGF can induce neovascularization but do not induce vascular permeability (Connolly et al., 1989; Murohara et al., 1998). An analysis of mice deficient in specific SFKs demonstrates no decrease in VEGF-dependent neovascularization but a complete ablation of its VP activity in *src<sup>-/-</sup>* or *yes<sup>-/-</sup>* mice, while *fyn<sup>-/-</sup>* mice show no such defect. While mice lacking Src show no VP response to VEGF, they do show a VP response to an inflammatory mediator. Therefore, multiple SFKs can serve as key signaling intermediates involved in VEGF-induced vascular proliferation, while the VP activity of this growth factor depends on Src or Yes in particular.

### Results

#### Src Activity Is Required for VEGF-, but Not bFGF-, Induced Angiogenesis

To establish whether endogenous Src activity was associated with growth factor-mediated angiogenesis, filter disks saturated with either bFGF or VEGF were placed on the CAM of 10-day-old chick embryos. This treatment is known to promote a robust angiogenic response as measured after 48–72 hr (Brooks et al., 1994a). Lysates of these CAMs were evaluated for Src activity by immunoprecipitating Src and measuring its ability to phosphorylate a GST-focal adhesion kinase (FAK) fusion protein in an in vitro kinase assay. At least a 2-fold increase in endogenous Src activity was detected in these lysates 120 min after the addition of either bFGF or VEGF to the CAM tissue (Figure 1A). Importantly, we observed a similar increase in Src activity 15 min after the addition of either growth factor (data not shown). To determine whether Src activity was required for angiogenesis, CAMs stimulated with either bFGF or VEGF were infected with an avian-specific retroviral vector (RCAS) containing a truncation mutant of Src lacking its C-terminal kinase domain (Src 251) (Kaplan et al., 1994). This Src 251 and similar truncation mutants have been shown to function as a dominant negative of multiple SFKs, thereby blocking signaling events downstream of growth factor receptors (Broome and Hunter, 1996; P. L. S., unpublished data). The RCAS retrovirus, when applied to CAM tissues, infects fibroblasts and endothelial cells proximal to the filter disk as determined by infecting CAMs with an RCAS-GFP vector and examination by

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**Figure 1. Activation of Endogenous Src Kinase Activity by bFGF and VEGF and the Effect of Kinase-Deleted Src on Angiogenesis In Vivo**

(A) Tissue extracts of 10-day-old chick CAMs were exposed to filter paper disks saturated with bFGF or VEGF (2  $\mu$ g/ml) for 2 hr. Endogenous Src was immunoprecipitated from equivalent amounts of total protein and subjected to an in vitro immune complex kinase assays with a FAK-GST fusion protein as a substrate, electrophoresed, and transferred to nitrocellulose. The relative fold increase in Src activity is indicated in *italics*. The above kinase assay blot was probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

(B) Chick CAMs (9 day) were exposed to filter paper disks saturated with RCAS-Src 251 (kinase deleted) or RCAS-GFP containing retroviruses or buffer for 20 hr and then incubated in the presence or absence of bFGF or VEGF for an additional 72 hr. Tissue extracts of these CAMs were examined for endogenous Src activity by in vitro immune complex kinase assay as described above using FAK-GST as a substrate.

(C) The level of angiogenesis was quantified in embryos incubated with RCAS-Src251 or RCAS-GFP followed by stimulation with either bFGF or VEGF as described above. Blood vessels were enumerated by counting blood vessel branch points in a double blinded manner. Each bar represents the mean  $\pm$  SEM of three replicates.

(D) Micrographs of representative CAMs were taken with an Olympus stereomicroscope. Scale bar, 350  $\mu$ m.

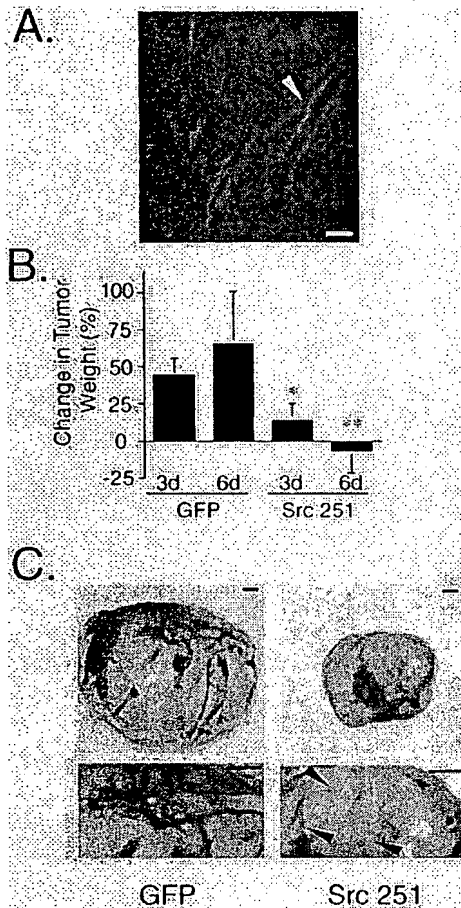
confocal microscopy (data not shown). Delivery of this kinase-deleted Src completely disrupted endogenous Src kinase activity in these tissues induced with either growth factor (Figure 1B).

To examine the role of Src in angiogenesis, CAMs stimulated with either bFGF or VEGF were infected with the Src 251-containing retrovirus. As shown in Figure 1C, angiogenesis, as measured 72 hr after stimulation with VEGF, was suppressed by delivery of Src 251; however, to our surprise, bFGF-induced angiogenesis was not affected. Importantly, equivalent levels of viral infection were detected in VEGF- and bFGF-stimulated CAMs as measured by epifluorescence and immunoblot analysis of GFP and Src 251, respectively (data not shown). The inhibition of VEGF-induced angiogenesis by kinase-deleted Src was likely due to a direct effect on endothelial cells, since VEGF is a known endothelial cell-specific mitogen. In addition, the failure of Src 251 to disrupt bFGF-induced angiogenesis indicates that the effects on VEGF-mediated angiogenesis are not due to general toxicity. Together, these results demonstrate that, while both bFGF and VEGF can activate Src kinase in these tissues, only VEGF-induced blood vessel formation required this activity. These findings support the recent

reports that VEGF and bFGF stimulate distinct pathways of angiogenesis (Friedlander et al., 1995; Ziche et al., 1997).

#### Suppression of Human Tumor Growth by Targeting the Tumor Vascular Compartment with Retroviral Delivery of Src 251

Tumor growth depends on angiogenesis (Weidner et al., 1991; Folkman and Shing, 1992; Brooks et al., 1994b). In fact, recent reports suggest that tumor growth is susceptible to the antiangiogenic effects of VEGF receptor antagonists (Kim et al., 1993). Therefore, experiments were designed to determine whether suppression of angiogenesis by delivery of kinase-deleted Src 251 would influence the growth of a human medulloblastoma (DAOY), a highly angiogenic tumor known to produce VEGF and very little bFGF (data not shown). This human tumor readily grows on the CAM and produces an active angiogenic response (Figure 2), allowing us to selectively target the tumor vasculature by using the avian-specific RCAS retrovirus, without infecting the human medulloblastoma cells. Delivery of RCAS containing Src 251 to preestablished medulloblastomas resulted in a selective expression of the virus in the tumor-associated



**Figure 2. Retroviral Delivery of RCAS-Src 251 to Human Tumors Growing on the Chick CAM Reverses Tumor Growth**

(A) Human DAOY medulloblastomas, which express VEGF, were grown on the CAM of chick embryos as described in the Experimental Procedures. Retrovirus containing RCAS-GFP or RCAS-Src 251 was topically applied to preestablished tumors of greater than 50 mg. A representative micrograph of a medulloblastoma tumor fragment infected with RCAS-GFP expressing GFP reveals exclusive expression in the tumor blood vessels (arrowhead) as detected by optical sectioning with a Bio-Rad 1024 laser confocal scanning microscope. Scale bar, 500  $\mu$ m.

(B) Tumors treated as above were allowed to grow for 3 or 6 days, after which they were resected and wet weights were determined. Data are expressed as the mean change in tumor weight (from the 50 mg tumor starting weight)  $\pm$  SEM of two replicates. RCAS-Src 251 had a significant impact on tumor growth after 3 days (\* $p$  < 0.002) and 6 days (\*\* $p$  < 0.05).

(C) Representative stereomicrographs of medulloblastoma tumors surgically removed from the embryos were taken with an Olympus stereomicroscope (scale bar, 350  $\mu$ m). (Lower panel) A high magnification micrograph of each tumor showing the vasculature in detail (scale bar, 350  $\mu$ m). The arrowhead indicates blood vessel disruption in RCAS-Src 251-treated tumors.

blood vessels (Figure 2A), which led to a complete suppression of tumor growth (Figure 2B). Importantly, the tumor-associated blood vessels in animals treated with virus containing Src 251 were severely disrupted and

fewer in number compared to the tumor vessels in control animals (Figure 2C). The fact that RCAS-GFP-infected tumors showed GFP localization only in the tumor vasculature suggests that the antitumor effects observed with retrovirally delivered Src 251 were due to its targeting and antiangiogenic properties.

#### Src Requirement for Endothelial Cell Survival during VEGF-, but Not bFGF-, Mediated Angiogenesis

Recent evidence suggests that growth factor receptors (Choi and Ballermann, 1995; Satake et al., 1998) and integrins (Meredith et al., 1993; Brooks et al., 1994a) promote survival of angiogenic endothelial cells. The fact that both growth factors and adhesion receptors also regulate Src activity prompted us to examine the role of Src in endothelial cell survival during angiogenesis. Furthermore, the Src 251 mutant has been found to induce apoptosis in selective cell types during bone development (P. L. S., L. Xing, and B. Boyce, unpublished data). CAMs stimulated with either bFGF or VEGF were infected with retrovirus containing Src 251, and cryostat sections of these tissues were examined for the presence of apoptotic cells. As shown in Figure 3A, delivery of Src 251 promoted extensive TUNEL staining among the factor VIII-related antigen (von Willebrand factor [vWf]) positive blood vessels in VEGF-, but not bFGF-, stimulated CAMS. In fact, minimal apoptosis was observed among other cell types in these CAMs (Figure 3), suggesting an endothelial cell-specific requirement for Src kinase activity for cell survival in VEGF-activated blood vessels. In a second series of experiments, retrovirus-infected CAMs stimulated with VEGF or bFGF were subjected to limited collagenase digestion to prepare a single cell suspension. These CAM-derived cells were shown to contain approximately 20%–50% endothelial cells (vWf positive) (Figures 3C and 3D) and analyzed for apoptosis by flow cytometric detection of annexin V-positive cells, an early apoptosis marker. As shown in Figure 3B, cells derived from VEGF-stimulated CAMs infected with Src 251 had significantly increased annexin V staining relative to cells from mock RCAS-GFP-infected CAMs treated with VEGF. In contrast, cells derived from mock-infected CAMs or those infected with RCAS-Src 251 and stimulated with bFGF exhibited little or no annexin V staining (data not shown). In addition, no annexin V staining was detected among cells derived from nonstimulated or bFGF-stimulated CAMs (data not shown). These data demonstrate that Src kinase activity is selectively required for endothelial cell survival during VEGF, but not bFGF-mediated angiogenesis in the CAM.

#### Selective Requirement for Src Kinase Activity in a Subcutaneous Murine Model of Angiogenesis

To further analyze the role of Src in angiogenesis, a murine model was employed. In this case, angiogenesis was induced by subcutaneous injection of growth factor-depleted Matrigel supplemented with either bFGF (400 ng/ml) or VEGF (400 ng/ml) in athymic wehi (nu/nu) adult mice and analyzed after 5 days (Passaniti et al., 1992). Angiogenesis was quantitated by removing and extracting the angiogenic tissue and then subjecting the



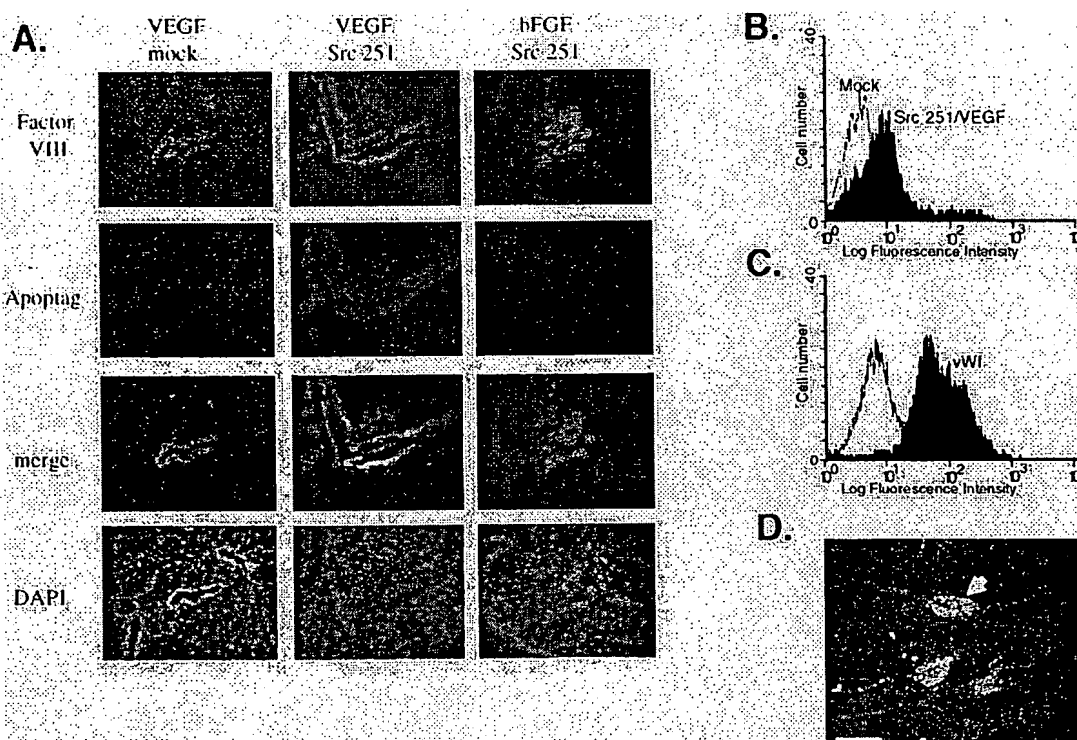


Figure 3. Apoptosis in VEGF-Stimulated Blood Vessels Expressing Src 251

(A) Immunolocalization of factor VIII-related antigen (von Willebrand factor), apoptag immunostaining of apoptotic cells, and nuclear staining with DAPI in cryosections of CAMs expressing RCAS-Src 251 or RCAS-GFP, after stimulation with bFGF or VEGF as described in Figure 1. The merge represents an overlay of the factor VIII staining and apoptag staining. The fluorescence from the GFP was not preserved in the fixation protocol used for the indirect immunofluorescence in these experiments. These micrographs were representative of blood vessel staining in duplicate samples. Scale bar, 50  $\mu$ m.

(B) Apoptotic cells were identified by annexin V staining of RCAS-Src 251-infected CAMs treated with VEGF and detected by flow cytometry. Collagenase-dissociated cells isolated from RCAS-Src 251- (black) or RCAS-GFP- (mock, white) infected CAMs treated with VEGF, as described in Figure 1, were incubated with annexin V. The fluorescence from the GFP was not detected in these assays, and the FACS profile was similar to untreated controls. The flow cytometry data for each experiment was representative of at least three replicates.

(C) Anti-vWf staining was detected with a FITC-labeled secondary antibody used to identify endothelial cells by flow cytometry, and this was compared to parallel collagenase-dissociated untreated CAM cells incubated without primary antibody.

(D) Immunolocalization of endogenous von Willebrand factor in collagenase-dissociated untreated permeabilized CAM cells (arrowhead) replated on 3  $\mu$ g/ml collagen and detected with a fluorescent secondary antibody (bar, 10  $\mu$ m).

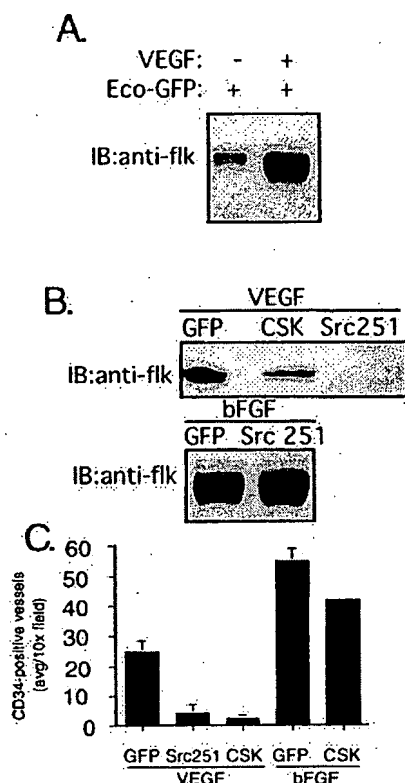
lysates to immunoblotting with a VEGF receptor antibody (flk-1) (Figure 4A) that is specific for endothelial cells. As observed in the chick, expression of the kinase-deleted Src 251 cDNA blocked VEGF-induced angiogenesis in this murine model while having no effect on bFGF-induced angiogenesis (Figure 4B). To establish the role of endogenous Src in this model, tissues were infected with a retrovirus expressing Csk that inhibits endogenous Src activity by phosphorylation of the C-terminal regulatory site (Nada et al., 1991). Expression of Csk blocked VEGF-, but not bFGF-, induced angiogenesis (Figure 4), confirming a role for endogenous Src activity in VEGF-mediated angiogenesis. Neovascularization of these virus-infected VEGF-stimulated tissues was confirmed by direct immunofluorescence with a FITC-conjugated anti-CD34 antibody (Figure 4) or an anti-flk-1 antibody (data not shown) and quantitated by enumerating

the number of positively stained CD34 blood vessels in each cryosection (Figure 4C).

#### The Effect of Intradermal Expression of VEGF in *src*<sup>-/-</sup> or *src*<sup>+/-</sup> Mice Ears

To extend the observations made in the chicken and mouse angiogenesis models, a direct genetic approach was employed to examine intradermal VEGF-induced angiogenesis in *src*<sup>-/-</sup> mice. We also considered the fact that VEGF both initiates new blood vessel growth and can promote vascular permeability (Senger et al., 1983; Ferrara and Davis-Smyth, 1997). Intradermal injections of adenovirus expressing a human VEGF cDNA were performed in the ear of *src*<sup>+/-</sup> and *src*<sup>-/-</sup>, while control  $\beta$ -galactosidase expressing adenovirus was injected into the opposite ear of each mouse. VEGF-dependent





**Figure 4.** Retroviral Delivery of Src 251 and Csk in a Subcutaneous Murine Angiogenesis Model

(A) Angiogenesis was induced by a subcutaneous injection of growth factor-depleted Matrigel containing saline or VEGF (400 ng/ml) with  $2 \times 10^6$  ecotropic packaging cells expressing GFP retrovirus in the flank of athymic wehi (nu/nu) mice and analyzed after 5 days of incubation. The neovascularization was quantitated by immunoblotting with a VEGF receptor antibody (flk-1) that is specific for endothelial cells.

(B) The effects of kinase-deleted Src 251, Csk, or GFP retrovirus on VEGF- (400 ng/ml) or bFGF- (400 ng/ml) induced angiogenesis was analyzed by immunoblotting the tissue lysates with an anti-flk-1 antibody.

(C) The effect of the Src 251- and Csk-expressing retroviruses on VEGF-induced neovascularization was quantified by enumerating the number of CD34 positive vessels in tissue cross sections by indirect immunofluorescence in triplicate random fields at  $20\times$  as described in the Experimental Procedures.

new blood vessel growth in *src*<sup>+/+</sup> ears was first detectable within 48 hr, and neovascularization was analyzed after 5 days (Figure 5A). There were identical viral expression levels in *src*<sup>+/+</sup> and *src*<sup>-/-</sup> as determined by X-gal staining of  $\beta$ -galactosidase-adenovirus injected ears (data not shown). In VEGF-injected *src*<sup>-/-</sup> ears, there was no significant decrease in angiogenesis (data not shown) as measured by counting branch points ( $p < 0.05$ ). However, the most apparent phenotype in these animals was the complete blockade in the vascular leakage compared to the VEGF-injected *src*<sup>+/+</sup> ears. Representative ears injected with VEGF are shown in Figure

5A, which confirms the extent of the vascular leakage in *src*<sup>+/+</sup> mice that is essentially absent in the *src*<sup>-/-</sup> mice. The vascular leakage in these animals suggested that the VP activity, which has been associated with angiogenesis in vivo (Dvorak et al., 1995), could be selectively disrupted in *pp60*<sup>src</sup>-deficient mice.

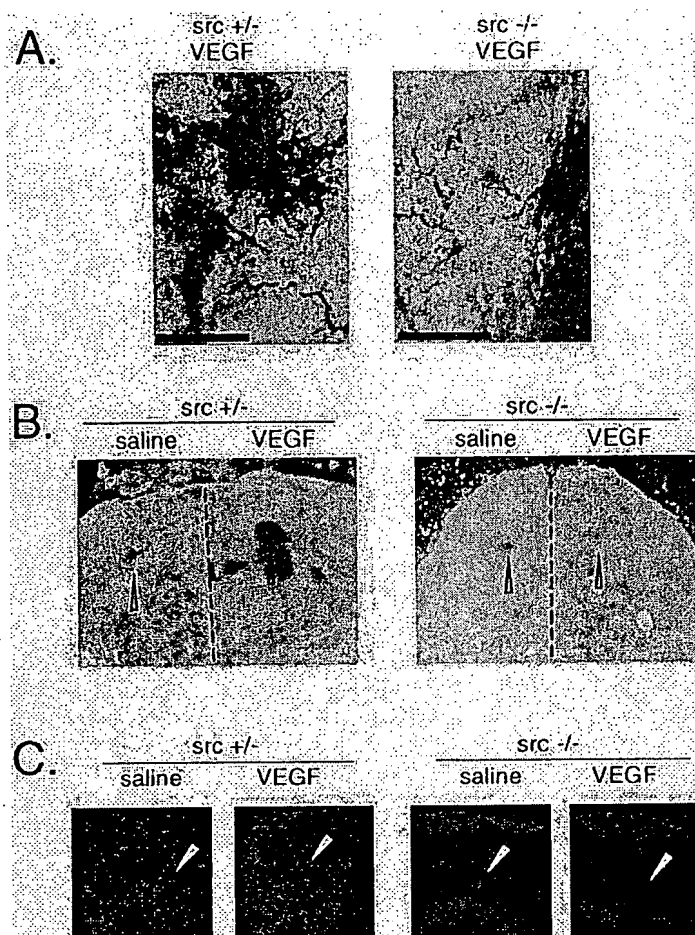
#### VEGF Fails to Compromise the Blood-Brain Barrier in Mice Lacking *pp60*<sup>src</sup>

The brain vasculature is characterized by a highly restrictive blood-brain barrier that prohibits small molecules from extravasating into the surrounding brain tissue. Tumor growth within the brain can compromise this barrier due in part to the production of angiogenic growth factors such as VEGF. Therefore, we examined the nature of the blood-brain barrier in *src*<sup>+/+</sup> or *src*<sup>-/-</sup> mice. In this case, VEGF or saline was stereotactically injected into the right or left hemisphere of the brain, respectively. All mice received systemic injections of Evan's blue to monitor VP activity. As shown in Figure 5B, vascular leakage of blood was localized to the VEGF-injected hemisphere in *src*<sup>+/+</sup> mice, but there a complete absence of vascular leakage in *src*<sup>-/-</sup> mice. This was also the case when examining the VP by measuring the accumulation of Evan's blue dye as detected by epifluorescence analysis of cryostat sections of these brains (Figure 5C). Thus, VEGF compromises the blood-brain barrier in a manner that depends on *pp60*<sup>src</sup>.

#### VEGF-Mediated VP, but Not Inflammation-Associated VP, Depends on *pp60*<sup>src</sup>

To further analyze and quantitate the effect of VEGF as a VP factor in *src*<sup>+/+</sup> or *src*<sup>-/-</sup> mice, we used the Miles assay (Miles and Miles, 1952) to quantitatively measure the vascular permeability in the skin of these animals. VEGF was injected intradermally in *src*<sup>+/+</sup> or *src*<sup>-/-</sup> mice that had received an intravenous systemic administration of Evan's blue dye. Within 15 min after injection of VEGF, there was a 3-fold increase in VP in *src*<sup>+/+</sup>. However, in *src*<sup>-/-</sup> mice, we observed no detectable VP activity (Figures 6A and 6B). Dye elution of the injected skin patches was quantitated and compared with control saline and bFGF (Figure 6B, left panel). bFGF or saline controls injected adjacent to the VEGF showed no significant increase in VP.

Vascular leakage/permeability is also known to occur during inflammation, which allows for the accumulation of serum-associated adhesive protein and inflammatory cells in tissues. In fact, inflammatory mediators themselves directly promote vascular leakage. Therefore, we tested one such inflammatory mediator, allyl isothiocyanate, also known as mustard oil (Inoue et al., 1997), in *src*<sup>+/+</sup> or *src*<sup>-/-</sup> mice for its capacity to produce VP. Unlike that observed in VEGF-stimulated *src*<sup>-/-</sup> animals, we detected no decrease in the VP produced by injection of the inflammatory mediator allyl isothiocyanate (Figure 6B, right panel). Thus, we conclude that Src plays a selective role in the VP activity induced with VEGF and does not influence VP associated with the inflammatory process.



**Figure 5. The Effect of VEGF-Induced Vascular Leakage in the Ears and Brains of *src*<sup>+/-</sup> and *src*<sup>-/-</sup> Mice**

(A) Gene delivery of the human VEGF cDNA in an adenovirus vector was injected intradermally in the right ear of *src*<sup>+/-</sup> or *src*<sup>-/-</sup> mice, and the neovascularization of the ears were photographed after 5 days of expression. Adenovirus expressing  $\beta$ -galactosidase was injected into the left ears as a negative control. Staining for  $\beta$ -galactosidase in these ears confirmed similar adenovirus expression in each genetic background. Scale bar, 1 mm;  $n = 4$ .

(B) VEGF or saline was stereotactically injected into the left or right frontal lobes, respectively, of *src*<sup>+/-</sup> or *src*<sup>-/-</sup>. After injection with Evan's blue and perfusion, the brains were removed and photographed with a stereoscope (6 $\times$ , final magnification; arrowhead, injection site).

(C) Cross sections of the above VEGF- or saline-injected brains from *src*<sup>+/-</sup> or *src*<sup>-/-</sup> mice were prepared and analyzed for VEGF-induced VP by confocal microscopy to visualize the fluorescence of the extravasated Evan's blue (6 $\times$ , final magnification; arrowhead, injection site).

#### VEGF-Mediated VP Activity Depends on Src and Yes but Not Fyn

We next tested the specificity of the Src requirement for VP by examining the VEGF-induced VP activity associated with SFKs such as Fyn or Yes, which, like Src, are known to be expressed in endothelial cells (Bull et al., 1994; Kiefer et al., 1994). In fact, we confirmed that these three SFKs were expressed equivalently in the aortas of wild-type mice (data not shown). Like *src*<sup>-/-</sup> mice, animals deficient in Yes were also defective in VEGF-induced VP (Figure 6C). However, to our surprise, mice lacking Fyn retained a high VP in response to VEGF that was not significantly different from control animals (Figure 6C). The disruption of VEGF-induced vascular permeability in *src*<sup>-/-</sup> or *yes*<sup>-/-</sup> mice demonstrates that the kinase activity of specific SFKs is essential for VEGF-mediated signaling event leading to VP activity but not angiogenesis.

#### Discussion

While multiple growth factors and adhesion events can promote angiogenesis, little is known regarding the sig-

naling events required for the growth of new blood vessels. In this report, evidence is provided that two angiogenic growth factors, bFGF and VEGF, initiate signaling pathways that can be distinguished based on their requirement for Src kinase activity. Even though both bFGF and VEGF led to increased Src activity in angiogenic tissues, only VEGF-induced angiogenesis depended on it. This was based on studies where kinase-deleted Src or Csk was retrovirally delivered to stimulated blood vessels. The use of intact Csk was important as it blocks the activity of endogenous Src rather than acting as a dominant-negative mutant like Src 251. Src activity was found to be required for the survival of VEGF-stimulated endothelial cells in vivo.

VEGF was originally described as a vascular permeability factor secreted by tumor cells (Senger et al., 1983). Using mice deficient for specific SFKs, we demonstrated that pp60<sup>src</sup> or pp62<sup>yes</sup> are essential for VEGF-induced VP, while its angiogenic activity was not significantly influenced in these animals. Moreover, animals deficient in Fyn show no loss of VP activity demonstrating that only certain SFKs are required to regulate VEGF-mediated VP activity. Importantly, all three of

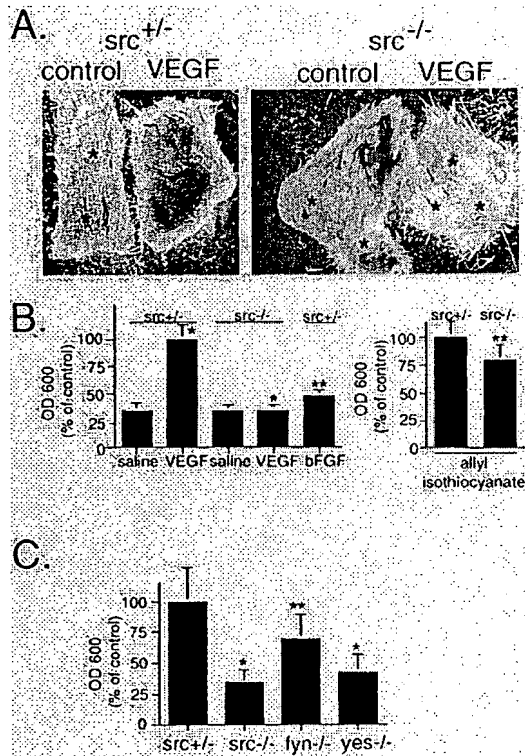


Figure 6. Mice Assay for Vascular Permeability of VEGF in the Skin of Mice Deficient in Src, Fyn, or Yes

(A) The vascular permeability properties of VEGF in the skin of *src*<sup>+/+</sup> (upper) or *src*<sup>-/-</sup> (lower) mice was determined by intradermal injection of saline or VEGF (400 ng) into mice that have been intravenously injected with Evan's blue dye. After 15 min, skin patches were photographed (scale bar, 1 mm). Arrowheads indicate the injection sites. (B) The regions surrounding the injection sites of the VEGF, bFGF, or saline were dissected, and the permeability quantitated by elution of the Evan's blue in formamide at 56°C for 24 hr, and the absorbance measured at 600 nm (left). The ability of an inflammation mediator (allyl isothiocyanate), known to induce inflammation-related VP, was tested in *src*<sup>+/+</sup> or *src*<sup>-/-</sup> mice (right).

(C) The ability of VEGF to induce VP was compared in *src*<sup>-/-</sup>, *fyn*<sup>-/-</sup>, or *yes*<sup>-/-</sup> mice in the Mice assay. Data for each of the Mice assays are expressed as the mean  $\pm$  SD of triplicate animals. *src*<sup>-/-</sup> and *yes*<sup>-/-</sup> VP defects compared to control animals were statistically significant (\**p* < 0.05, paired *t* test), whereas the VP defects in neither the VEGF-treated *fyn*<sup>-/-</sup> mice nor the allyl isothiocyanate-treated *src*<sup>-/-</sup> mice were statistically significant (\*\**p* < 0.05).

these SFKs were shown to be equivalently expressed in the aortas, skin, and brain of wild-type mice and are known to be expressed in endothelial cells (Bull et al., 1994; Kiefer et al., 1994). To our surprise, inflammation-induced VP was shown to be independent of Src kinase in these mice, suggesting that the VP activity induced during inflammation and that induced upon VEGF stimulation are regulated by distinct signaling pathways.

Mice lacking pp60<sup>csrc</sup> and pp62<sup>cyes</sup> show apparently normal vascular development, even though mice lacking VEGF or its receptor die during development. Thus, VEGF-induced VP activity is not required for development. However, it may play a role in wound repair or

other postnatal process. Interestingly, mice lacking the combination of Src, Yes, and Fyn or the VEGF receptor show embryonic lethality by day 9.5, a time during development that is characterized by active vasculogenesis (Fong et al., 1995; Shalaby et al., 1995). This, together with the fact that mice lacking individual SFKs develop normal appearing blood vessels, suggests that compensation can take place among these SFKs. This is supported by our observation that suppression of Src kinase activity in general by Csk or Src251 suppressed neovascularization in mice or chick embryos in response to VEGF while individual SFK knockouts develop normally.

Evidence provided in this study demonstrates that VEGF and bFGF potentiate somewhat different biological and biochemical effects during the early stages of angiogenesis. There may be a physiological rationale for the existence of two angiogenic pathways. For example, blood vessels in various organs may differ with respect to distinct ECM-associated adhesive proteins and/or growth factors. Neovascularization in the retina has been linked to VEGF expression (D'Amore, 1994; Miller et al., 1994), while that induced during cutaneous wound repair has been associated with bFGF (Takenaka et al., 1997). This may allow endothelial cells to meet the specific needs of a given tissue depending on local requirements for nutrients, oxygen, or waste elimination. After hypoxic injury, VEGF levels are known to rise immediately (reviewed in Ferrara and Davis-Smyth, 1997). Perhaps this hypoxic response facilitates an immediate increased oxygenation by providing local vascular leakage prior to the actual formation of a new vascular network. This would predict that adult pp60<sup>csrc</sup> or pp62<sup>cyes</sup>-deficient mice may be less capable of restoring oxygenation to damaged or hypoxic tissue. In fact, we noted that stereotactic injection of VEGF in the brain could compromise the blood-brain barrier in control animals. However, animals deficient in pp60<sup>csrc</sup> showed no breakdown of the blood-brain barrier.

VEGF is an angiogenic growth factor in many tumors. In fact, an anti-VEGF antibody (Kim et al., 1993) that blocks tumor growth in mice is being evaluated clinically in patients with late-stage cancer. Given the strong association between VEGF and tumor angiogenesis, our results may provide another approach to disrupt the growth of tumors. Thus, by using an avian-specific retrovirus, we were able to specifically target the chick vasculature of a growing human medulloblastoma. Even though the tumor cells remained uninfected by the retrovirus, we observed suppressed tumor growth demonstrating the potential therapeutic efficacy of this approach.

In a combination of experiments using retrovirally delivered mutant Src and Csk as well as a direct analyses of *src*<sup>-/-</sup> mice, we provide evidence that the Src tyrosine kinase family distinguishes two pathways of angiogenesis. During VEGF-induced angiogenesis, SFK activity contributes to endothelial cell survival. Furthermore, the VEGF-induced VP is dependent on SFKs, Src, or Yes, but not Fyn, and the VP response is specific for VEGF in contrast to inflammation-induced VP. Therefore, while SFKs serve compensatory roles during embryogenesis and angiogenesis, VEGF-, but not bFGF-, mediated angiogenesis requires Src kinase activity for endothelial

cell survival, whereas VP activity of VEGF depends on the SFKs, Src, or Yes.

#### Experimental Procedures

##### Antibodies and Reagents

A rabbit polyclonal antibody raised against amino acids 3–18 of human Src (N-16; Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoprecipitation in *in vitro* kinase assays, and monoclonal antibody against avian pp60<sup>src</sup> (Upstate Biotechnology, Lake Placid, NY) was used for Western blotting as a loading control for the kinase assays. The Src constructs were obtained from Dr. H. Varmus (NIH). FAK–GST fusion protein was from Dr. D. Schlaepfer (The Scripps Research Institute [TSRI]), the DF-1 virus producer cell line was from Dr. D. Foster (University of Minnesota), the DAOY medulloblastoma cell line from Dr. W. Laug (Children's Hospital, USC, Los Angeles), RCAS–GFP was from Dr. C. Cepko (Harvard), and bFGF was kindly provided by Dr. J. Abraham (Scios, Mountain View, CA). All other reagents and media were from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

##### Src Constructs and Retroviruses

For the studies in the chick embryo, the replication competent RCASBP(A) (Hughes et al., 1987) retrovirus was used to express mutant Src cDNAs subcloned as NotI–ClaI. These constructs were transfected into the chicken immortalized fibroblast line, DF-1. Viral supernatants were collected from DF-1 producer cell lines in serum-free CLM media. Viral supernatants were concentrated by ultracentrifugation at 4°C for 2 hr at 22,000 rpm, and the pellets were resuspended in 1/100 the original volume in serum-free media with a titer of at least 10<sup>8</sup> Lu (infectious units)/ml and stored at –80°C.

For the retrovirus studies in the subcutaneous murine matrigel angiogenesis assay, GFP, kinase-deleted Src 251, and Csk cDNA was subcloned into the replication-defective murine Moloney retrovirus (pLNCX) vector. These constructs were transiently transfected into the ecotropic producer line to generate cell-free titers of 10<sup>5</sup>–10<sup>6</sup> Lu/ml. Therefore, to increase the effective titer over the 5 day time course of the angiogenesis assay in the matrigel plug, the virus-packaging cells expressing the appropriate construct were included along in the Matrigel to increase the retrovirus infection levels.

##### VEGF Adenovirus

Recombinant VEGF adenovirus was generated by cloning the human VEGF cDNA from a human placenta cDNA library into pAd/CI (J. L. A. Reddy, and D. A. C., unpublished data) and cotransfecting with pJM17 into an E1 transcomplementing 293 cell line as previously described (Bett et al., 1994). High titer virus was isolated, purified, and titered to 10<sup>11</sup> pfu/ml as previously described (Chang et al., 1995). High titer clones were selected based on their expression of soluble VEGF secreted into the media of COS-7, endothelial cells, and in chick CAMs infected with the VEGF adenovirus (data not shown).

##### Chick Embryo Treatments

Fertilized chick embryos (standard pathogen free grade; SPAFAS, Preston, CT) were prepared, and the CAM was exposed as previously described (Eliceiri et al., 1998). For growth factor–only experiments, cortisone acetate-soaked filter disks were soaked with 250 ng of bFGF or VEGF for 2 hr before harvest. For virus experiments on the CAM, disks were soaked in 20  $\mu$ l of viral stock per disk. These disks were applied to the CAM of 9 day chick embryos and incubated at 37°C for 24 hr. Then, either serum-free media or growth factors were added at a concentration of 5  $\mu$ g/ml to the CAM in 20  $\mu$ l of the virus stock as an additional boost of virus to the CAM tissue and incubated for an additional 72 hr. CAM assays were quantitated by counting branch points as described previously (Eliceiri et al., 1998) in triplicate samples in a double blind manner.

##### Immunoprecipitation and Immunoblotting

CAM tissues were homogenized in a RIPA lysis buffer, used for immunoprecipitations or immunoblots as previously described (Eliceiri et al., 1998). Anti-Src and anti-flk1 antibodies used in immunoblots were detected with horseradish peroxidase-conjugated

goat anti-mouse secondary antibodies as previously described (Eliceiri et al., 1998).

##### In Vitro Kinase Assay for Src Kinase

The kinase activity of endogenous Src kinase was assayed by the ability of immunopurified Src to phosphorylate a FAK–GST fusion protein in an *in vitro* assay. Src was immunoprecipitated as described above and subjected to a kinase assay, and the samples were analyzed by 15% SDS-PAGE and quantitated as described previously (Eliceiri et al., 1998).

##### Immunostaining and Annexin V Labeling of Apoptotic Cells

Cryosections of CAMs treated with RCAS–GFP or RCAS–Src 251 treated with bFGF or VEGF were analyzed for apoptotic cells using the Apoptag kit (Oncor, Gaithersburg, MD). Sections were also immunostained with a rabbit polyclonal anti-vWF (Biogenix, San Ramon, CA) and counterstained with 1  $\mu$ g/ml DAPI. Fluorescent images were captured with a cooled CCD camera (Roper, Trenton, NJ), and the fluorescent images were processed and exposure matched between experimental treatments as previously described (Eliceiri et al., 1998).

To measure the apoptotic index of retrovirus-infected CAM tissues, FITC-conjugated annexin V (Clontech, Palo Alto, CA) was used to stain cell suspensions, and the washed cells were analyzed by flow cytometry. Cell suspensions of CAM cells were prepared from mock- or virus-infected CAMs by digestion with 0.1% (w/v) collagenase type IV (Worthington Biochemicals, Lakewood, NJ) in RPMI 1640 of minced CAM tissue rocking for 1 hr at 37°C as previously described (Brooks et al., 1994b) and filtered through 100  $\mu$ m nylon mesh (Becton Dickinson, Fountain Lakes, NJ). Fluorescence was measured with a FACscan flow cytometer (Becton Dickinson) to count 10,000 cells.

Measurement of vWf staining by FACS was performed with parallel collagenase digested CAM tissue cell preparations, that were fixed in 1.6% paraformaldehyde, permeabilized in 70% ethanol, incubated the anti-vWf antibody, and detected with a FITC-conjugated secondary antibody.

##### Tumor Growth Assay

The 3 and 6 day DAOY medulloblastoma tumor growth assays were performed in the chick CAM essentially as previously described (Brooks et al., 1994b). DAOY cells ( $5 \times 10^4$ ) were seeded on the CAM of a 10 day embryo. After 7 days, 50 mg tumor fragments were dissected and reseeded on another 10 day embryo and incubated for another 3 or 6 days with the topical application (25  $\mu$ l) of either control RCAS–GFP retrovirus, RCAS–Src 251, or mock treatment. Tumor resections and weighing were performed in a double blind manner removing only the easily definable solid tumor mass (Brooks et al., 1994b). The wet tumor weights after 3 or 6 days were compared with initial weight, and the percent change of tumor weight was determined for each group.

##### Immunofluorescence and Microscopy

Cryosections of the plugs were also subjected to immunofluorescent staining with an anti-CD34 antibody or an anti-flk antibody, photographed, and quantitated as described above for the CAM angiogenesis assays.

Whole-mount direct fluorescence of RCAS–GFP-infected tumor fragment was accomplished by dissecting a tumor fragment and imaging the unfixed tissue directly on a slide with a laser confocal microscope (MRC 1024; Bio-Rad, Hercules, CA).

##### Murine Matrigel Angiogenesis Assay

Growth factor–depleted Matrigel (Becton Dickinson) (400  $\mu$ l) supplemented with PBS, bFGF (400 ng/ml), or VEGF (400 ng/ml) (Passaniti et al., 1992) and murine-specific ecotropic packaging cells ( $\phi$ NX-Eco; G. Nolan, Stanford) producing retrovirus expressing GFP, Src 251, or Csk cDNAs was injected subcutaneously in 6-week-old male athymic wehi (nu/nu) mice. The plugs remained palpable for 5 days, facilitating a direct resection of the plug for further analysis by immunoblotting of plug homogenates or immunostaining of plug cryosections. The accuracy of the quantitative methods was confirmed by spectrophotometric analysis of homogenates of plugs from animals

that had been intravenously injected with FITC-labeled lectin (J. D. H. and D. A. C., unpublished data).

**Intradermal Ear Injections and Miles Assay**  
pp60<sup>src</sup>, pp59<sup>lck</sup>, and pp62<sup>src</sup>-deficient mice (129/SvEv × C57Bl6/J) were generated as previously described (Soriano et al., 1991) and were the generous gift of Drs. P. Soriano and P. Stein. Additional stocks were obtained from Jackson Labs. Mouse ears were injected intradermally (Eriksson et al., 1980) with 5 µl of adenovirus expressing either VEGF or β-galactosidase and the ears photographed after 5 days with a stereoscope.

The Miles assay (Miles and Miles, 1952) was adapted for mice by injecting 10 µl of VEGF (400 ng/ml), allyl isothiocyanate (mustard oil, 20% v/v in mineral oil), or saline intradermally into mice that had previously been intravenously injected with 100 µl of 0.5% Evan's blue. After 15 min, the skin patches were dissected, photographed, and eluted at 56°C with formalin and quantitated with a spectrophotometer (OD<sub>660</sub>).

#### Intracerebral Injection and Determination of Blood-Brain Barrier Disruption

Saline or VEGF (200 ng in 2 µl) was injected stereotactically into the left or right frontal lobe 92 mm to the left/right of the midline, 0.5 mm rostral from bregma, and 3 mm in depth from the dura, respectively. The animals received an Evan's blue solution intravenously 30 min after injection, as described above. After an additional 30 min, the mice were perfused and the brains were removed. Evan's blue fluorescence was observed using confocal laser microscopy of fresh unfixed cryosections of the brain.

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## Development of Time-Resolved Immunofluorometric Assay of Vascular Permeability Factor

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We describe a two-site time-resolved immunofluorometric assay for guinea pig vascular permeability factor (VPF) for quantifying VPF in different biological fluids. Antibody against the carboxy terminus (C-IgG) is immobilized on microtiter wells, and antibody against the amino terminus (N-IgG) is labeled with  $\text{Eu}^{3+}$ -chelate. Line 10 tumor culture medium, known to be rich in VPF, is assayed in a two-step incubation. Bound  $\text{Eu}^{3+}$  is then quantified by dissociation into a fluorescent enhancement solution, with measurement of the time-resolved fluorescence. The analytical sensitivity is 0.35 VPF unit, and the intra-assay CV is about 20%. The assay is specific for VPF, because pre-treatment with the appropriate C- or N-peptide, or pre-extraction of VPF, greatly decreases fluorescence. The VPF immunoassay is highly correlated ( $r^2 = 0.94$ ) with the Miles permeability assay, the classical bioassay of VPF. In addition, the immunofluorometric assay is about 30-fold more sensitive than the Miles assay.

**Additional Keyphrases:** vascular endothelial growth factor · angiogenesis · line 10 tumor culture medium · bioassay compared

Vascular permeability factor (VPF), which is secreted by various tumor cells, is a highly conserved protein ( $M_r$  34 000–42 000) with potent vascular permeability-enhancing activity that causes accumulation of ascites fluid associated with tumor growth (1, 2).<sup>1</sup> In addition, recent studies have shown that VPF is similar or identical to vascular endothelial growth factor (VEGF), a mitogen specific for endothelial cells (3–7). Thus, tumor-secreted VPF (VEGF) may promote tumor angiogenesis directly by its mitogenic activity for endothelium. VPF (VEGF) may also elicit angiogenesis indirectly by its vascular permeability effect, which causes extravasation of plasma proteins, including fibrinogen, and deposition of an extravascular fibrin gel, which provokes ingrowth of vascular endothelial cells (8). Currently, the precise role of VPF (VEGF) in the pathogenesis of solid tumor growth is an area of intense investigation. Important to this investigation is the development of a simple, sensitive, and specific assay for VPF, preferably one that can be used for assaying VPF in various

biological fluids and tissue homogenates.

VPF was first measured by using the Miles assay (9), which measures the extravasation of intravenously injected Evans Blue dye into the dermis of guinea pigs in response to intradermal injections of VPF (1). The amount of accumulated dye can be quantified by extraction and measuring the absorbance at 620 nm (10). The Miles assay has been used widely to detect VPF in cell-free culture medium of tumor cells as well as in tumor ascites fluid (1, 2). However, this assay is not specific because it will detect permeability changes in response to other inflammatory mediators besides VPF. Also, fluids from different animal species cannot be used because foreign proteins commonly elicit nonspecific permeability changes, leading to a false-positive Miles test.

We report here a sensitive and specific immunofluorometric assay for detecting VPF. Antibodies raised against the C-terminus of VPF (C-IgG) were immobilized on microtiter wells and served as the "capture" antibody. Antibodies raised against the amino terminus of VPF (N-IgG) were labeled with  $\text{Eu}^{3+}$ -chelate and served as the "detector" antibody. In the presence of VPF, a "sandwich" configuration is formed. After the final wash step, bound  $\text{Eu}^{3+}$  is dissociated in the presence of  $\beta$ -diketone, forming a highly fluorescent chelate that can be read in a time-resolved fluorometer. This approach is termed "dissociation enhanced lanthanide fluoroimmunoassay," or DELFIA (11–14). The reagents and equipment required are commercially available.

### Materials and Methods

**Reagents.** DELFIA  $\text{Eu}^{3+}$ -labeling kits were purchased from Pharmacia-LKB Nuclear, Inc. (Gaithersburg, MD). Each kit contained 0.2 mg of labeling reagent [ $\text{N}^1$ -(*p*-isothiocyanatobenzyl)-diethylenetriamine- $\text{N}^1, \text{N}^2, \text{N}^3, \text{N}^3$ -tetraacetate- $\text{Eu}^{3+}$ ], a 100 nmol/L  $\text{Eu}^{3+}$  standard, highly purified bovine serum albumin (BSA; 75 g/L in Tris · HCl, pH 7.8 plus  $\text{NaN}_3$ , 0.5 g/L) stabilizer, enhancement solution (per liter, 15  $\mu\text{mol}$  of 2-naphthyltrifluoroacetone, 50  $\mu\text{mol}$  of tri-*n*-octylphosphine oxide, 100 mmol of acetic acid, 6.8 mmol of potassium hydrogen phthalate, and 1.0 g of Triton X-100 detergent), assay buffer (Tris · HCl solution, pH 7.8, containing BSA, bovine gamma globulin, Tween 40, diethylenetriaminepentaacetic acid, and  $\text{NaN}_3$ , 0.5 g/L), and wash concentrate solution (25-fold concentration of Tris · HCl/NaCl, pH 7.8, plus Tween 20) (11–14). PD-10 columns, Sepharose CL-6B, and Sephadex G-50 were from Pharmacia LKB Biotechnology (Piscataway, NJ). Macrosolute concentrators were from Amicon (Danvers, MA). Maxisorp microtiter plates and strips (96-well) were obtained from Nunc

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<sup>1</sup> Nonstandard abbreviations: VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; C-IgG, antibody against carboxy terminus of VPF; N-IgG, antibody against amino terminus of VPF; ELISA, enzyme-linked immunosorbent assay; and BSA, bovine serum albumin.

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Inc. (Naperville, IL). Serum-free medium (HL-1) was purchased from Ventrex Labs. Inc. (Portland, ME). Hemoglobin crystals were from Sigma Chemical Co. (St. Louis, MO). The GammaGone IgG removal device was from Genex Corp. (Gaithersburg, MD).

**Buffers.** The labeling buffer was 50 mmol/L  $\text{NaHCO}_3$ , pH 8.5, containing NaCl, 9 g/L. The elution buffer was 50 mmol/L Tris · HCl, pH 7.8, containing 9 g of NaCl and 0.5 g of  $\text{NaN}_3$  per liter. The coating buffer contained phosphate-buffered saline, pH 7.0, and the blocking reagent was 30 g/L hemoglobin solution.

**Polyclonal antibodies.** Polyclonal antibodies were raised against two synthetic peptides that correspond to the N- and C-termini of guinea pig VPF (designated N-IgG, C-IgG, respectively). In the single letter code, the 25-amino acid sequence of the N-terminus is APMAE-GEQKPREVVVKFMDVYKRSYC (15), and the 20-amino acid sequence of the C-terminus is YKARQLELNERT-CRCDKPRR (4). The C-terminal peptide was synthesized by Multiple Peptide Systems (San Diego, CA), and both peptides were used for generation of antibodies in rabbits as described (15), except that the C-terminal peptide was coupled to keyhole limpet hemocyanin with bis-diazo benzidine. The antibodies (N-IgG and C-IgG) were affinity-purified from rabbit antisera by using the respective peptides coupled to CNBr-Sepharose (Pharmacia LKB). Bound antibodies were eluted from Sepharose-peptide columns with 0.1 mol/L glycine, pH 2.5, and the activity against each peptide was determined by an ELISA (16). Briefly, we used a 1 g/L solution of peptide in 10 mmol/L NaCl and 10 mmol/L Tris, pH 8.5, to coat a 96-well microtiter plate. After blocking with normal human serum (100 mL/L) in phosphate-buffered saline, we added the respective anti-peptide IgG solution (200-, 2000-, or 10 000-fold dilution). Antibody binding was detected with a peroxidase-labeled goat anti-rabbit antibody (Kirkegaard and Perry Labs. Inc., Gaithersburg, MD) with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] as the enzyme substrate. Color development was determined with a THERMOMax microplate reader at 405 nm (Molecular Devices, Menlo Park, CA). All affinity-purified IgG preparations retained strong anti-peptide activities, even at 10 000-fold dilution. Moreover, both N-IgG and C-IgG (when bound to Protein A-Sepharose) adsorbed VPF efficiently from solution, as determined with the Miles vessel permeability assay (T. Sioussat et al., manuscript in preparation).

**$\text{Eu}^{3+}$ -labeling of N-IgG.** We performed  $\text{Eu}^{3+}$ -labeling of the affinity-purified N-IgG according to the DELFIA kit protocol with some modifications and pooled and concentrated the affinity-purified antisera to ~0.5 g/L, using an Amicon Macrosolute concentrator. The PD-10 column was pre-equilibrated with 40 mL of labeling buffer, and 2 mL of the antisera (0.5 g/L) was loaded on the column. We rinsed the column with labeling buffer, collected 1.0-mL fractions, and measured the absorbance at 280 nm with a Model U-2000 spectrophotometer (Hitachi Instruments Inc., Danbury, CT 06810). Fractions corresponding to peak absorbance were

pooled, and concentrated to ~1 mL, which typically contained 1.5 g/L IgG concentration (we used an absorptivity value of 1.34 for 1 g/L of IgG solution to calculate IgG concentration). We added 1.0 mL of the IgG solution to 0.2 mg of labeling reagent (containing the  $\text{Eu}^{3+}$ -chelate) and mixed it gently on a rotator for 16 h at room temperature.

**Purification of  $\text{Eu}^{3+}$ -labeled IgG.** Sepharose CL-6B was poured into a 1.5 × 30 cm column to a height of 18 cm. Next, we added pre-swollen Sephadex G-50 to a height of 28 cm and equilibrated the column with 180 mL of elution buffer. The  $\text{Eu}^{3+}$ -labeled IgG reaction mixture was added and fractionated on this column. We rinsed the column with elution buffer, collected 60 1-mL fractions, and measured their absorbance at 280 nm. We diluted a small aliquot of each fraction 10 000-fold with enhancement solution and determined the fluorescence with a 1232 DELFIA time-resolved fluorometer (Pharmacia Diagnostics, Fairfield, NJ), in which a pulsed xenon flash at 340 nm and electronic gating were used to detect fluorescence at 613 nm between 400 and 800  $\mu\text{s}$  after the excitation flash.

**Characterization of  $\text{Eu}^{3+}$ -labeled N-IgG.** Fractions corresponding to peak IgG absorbance (280 nm) and fluorescence were pooled (usually, fractions 25 to 33) and the resulting absorbance (280 nm) and fluorescence (10 000-fold dilution) were determined. The yield of  $\text{Eu}^{3+}$ /IgG was calculated as described in the DELFIA kit protocol (typically, 10  $\text{Eu}^{3+}$ /IgG). To increase the stability of the  $\text{Eu}^{3+}$ -labeled N-IgG, purified BSA was added to a final concentration of 1.0 g/L.

**Coating of microtiter strips.** We added 50  $\mu\text{L}$  of a 50-fold dilution of C-IgG (stock concentration of 0.64 g/L in phosphate-buffered saline) to each well of the microtiter strips, and incubated the plate overnight at 4 °C on a shaker. Thereafter, we washed the wells six times with DELFIA wash buffer, and blocked by incubation with 30 g/L hemoglobin solution at 20 °C for 2 h with gentle shaking. Plates were washed six times with DELFIA wash buffer before use.

**Line 10 cell cultures.** Line 10 tumor cells from guinea pig were grown as suspension cultures in serum-free defined medium HL-1 as described previously (17). Conditioned line 10 medium, which contains large amounts of VPF, was centrifuged and frozen at -70 °C to serve as calibrators.

**Immunoassay procedure.** We used freshly coated microtiter strips on the same day to assay VPF. We added 50  $\mu\text{L}$  of various dilutions of line 10 conditioned media (using HL-1 medium as the diluent) to each well and incubated at 20 °C for 2 h with gentle shaking. After six washes with wash buffer, we added 50  $\mu\text{L}$  of  $\text{Eu}^{3+}$ -labeled N-IgG (diluted appropriately in assay buffer), incubated for another 2 h at 20 °C, and again washed six times. Finally, we dispensed 200  $\mu\text{L}$  of enhancement solution into each well, and after 5 min of gentle shaking, read the absorbance of the plate in the 1232 DELFIA fluorometer.

**Miles vessel permeability assay.** We assayed the bio-



activity of VPF in the line 10 culture medium preparations, using the Miles assay as described previously (1, 17).

## Results

**Preparation and purification of  $\text{Eu}^{3+}$ -labeled N-IgG.**  $\text{Eu}^{3+}$  labeling of affinity-purified antibodies directed against the N-terminal region of VPF was performed as described in *Materials and Methods*. The Sepharose 6B/Sephadex G-50 chromatographic profile in Figure 1 shows two distinct peaks. The first peak (I) corresponds to  $\text{Eu}^{3+}$ -labeled N-IgG, and the second peak (II) represents unreacted  $\text{Eu}^{3+}$ -chelate. For this reason, we showed in a separate experiment that >90% of the fluorescence associated with peak I could be removed by an IgG-removing device (Gammagone), indicating that peak I comprised mainly  $\text{Eu}^{3+}$ -labeled N-IgG. We pooled fractions 25–33, corresponding to  $\text{Eu}^{3+}$ -labeled N-IgG, and determined the corrected protein concentration to be 115 mg/L (using the absorptivity value of 1.34 mentioned above, with corrections for absorbance of the thiourea bonds of  $\sim 0.008 \text{ A}$  per  $\mu\text{mol/L}$ ). We calculated the specific activity of the  $\text{Eu}^{3+}$ -labeled N-IgG to be  $\sim 10 \text{ Eu}^{3+}/\text{IgG}$ , using a  $1 \text{ nmol/L}$   $\text{Eu}^{3+}$  standard as described in the DELFIA kit protocol.

**Optimization of VPF immunoassay.** To determine the optimal dilution of  $\text{Eu}^{3+}$ -N-IgG, we studied the effect of various amounts of N-IgG on the VPF binding curve. Microtiter plate wells were immobilized with a constant amount (225 ng/well) of C-IgG. Because pure VPF is not available, we used line 10 tumor cell conditioned medium, which is rich in VPF (17), to standardize the assay. We used the same lot of line 10 conditioned medium in all experiments. The concentration of VPF was expressed in arbitrary units, i.e., 100 units is defined as the amount of VPF in our batch of undiluted line 10 tumor cell conditioned medium. We arbitrarily

defined "signal" as the fluorescence obtained with undiluted line 10 conditioned medium, and "noise" as the nonspecific fluorescence associated with HL-1 medium (0 unit). Thus, the signal-to-noise ratio is defined as  $\text{fluorescence}_{100 \text{ units}}/\text{fluorescence}_{0 \text{ unit}}$ . The effect of varying the N-IgG dilution (from five- to 50-fold) is shown in Figure 2A. We determined that 50-fold diluted N-IgG gave a maximal signal-to-noise ratio of 83 (Figure 2B).

In a separate experiment, we studied the effect of varying the C-IgG dilution, keeping  $\text{Eu}^{3+}$ -N-IgG constant at 115 ng/well. As shown in Figure 3, we obtained a maximal signal-to-noise ratio of 89 with 30-fold diluted C-IgG (1000 ng/well). However, because of our limited supply of C-IgG, we decided to use a 50-fold dilution of C-IgG (640 ng/well) to coat the microtiter wells; at this concentration, the signal-to-noise ratio was close to maximal at 80. For all subsequent experiments, we coated microtiter plate wells with 50-fold dilution of C-IgG, and bound VPF was detected with 50-fold dilution of  $\text{Eu}^{3+}$ -N-IgG.

**Sensitivity and intra-assay CV of VPF immunoassay.** To assess the analytical sensitivity of the VPF assay, we prepared line 10 conditioned medium corresponding to 0.25 unit, 0.50 unit, and 1.00 unit by diluting with HL-1 medium, then assayed these 10 times. HL-1 medium devoid of VPF served as the zero standard. The sensitivity or minimal detectable dose (defined as +2 SD above the zero standard), determined by extrapolation from the standard curve, was  $\sim 0.35$  unit (Figure 4A). The intra-assay CV was <20% at 0.50 unit (Figure 4B).

**Specificity of VPF immunoassay.** Because the format of this assay depended on C-IgG as the capture antibody and  $\text{Eu}^{3+}$ -N-IgG as the detector antibody, we used peptides corresponding to the N- and C-termini of VPF to demonstrate the assay specificity. As shown in Figure 5, inclusion of C-VPF, N-VPF, or both peptides in the assay inhibited the binding of VPF in line 10 medium by  $\sim 80\%$ . In addition, when VPF was selectively removed from line 10 conditioned medium (by unlabeled N-IgG followed by incubation with Protein A-Sepharose and

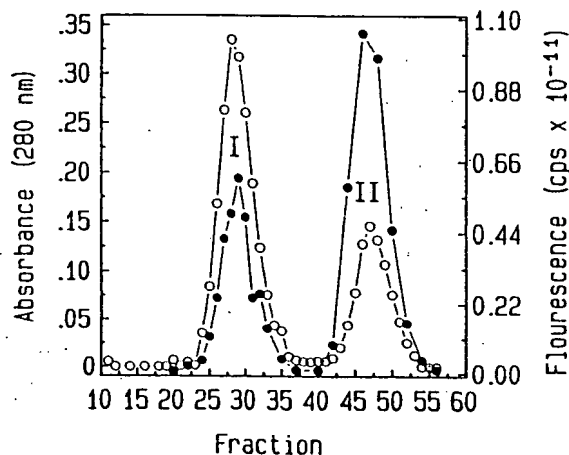


Fig. 1. Chromatographic profile of  $\text{Eu}^{3+}$ -labeled N-IgG

Absorbance at 280 nm (○) and fluorescence (●) were determined on fractions collected from a Sepharose 6B/Sephadex G-50 column. Each fraction was diluted 10 000-fold before fluorescence measurements, and results were expressed as total counts/s. Peak I denotes the  $\text{Eu}^{3+}$ -labeled N-IgG and peak II represents the free  $\text{Eu}^{3+}$ -chelate. Typical labeling yield is  $\sim 10 \text{ Eu}^{3+}$  per IgG molecule

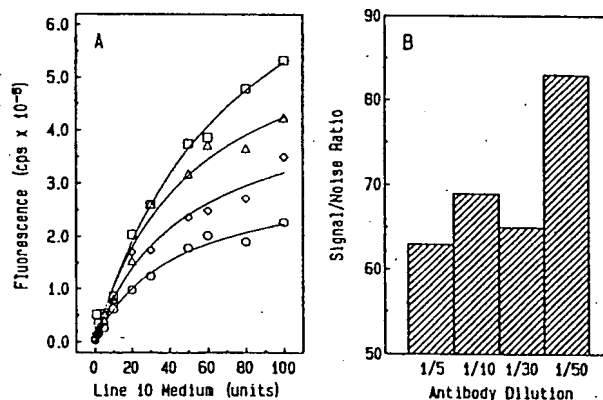


Fig. 2. Optimization of  $\text{Eu}^{3+}$ -labeled N-IgG

$\text{Eu}^{3+}$ -labeled N-IgG was used at various titers (at constant C-IgG) to determine the optimal titer. Signal-to-noise ratio is arbitrarily defined as  $\text{fluorescence}_{100 \text{ units}}/\text{fluorescence}_{0 \text{ unit}}$ . A: calibration curves at 50-fold (○), 30-fold (△), 10-fold (◇), and fivefold (□) dilution of N-IgG. B: effect of antibody dilutions on the signal-to-noise ratio

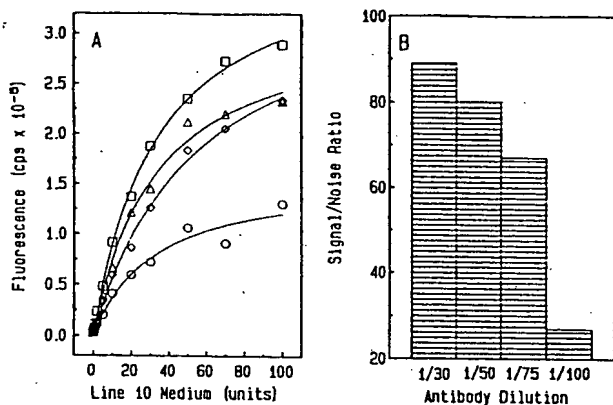


Fig. 3. Optimization of C-IgG

A: C-IgG was coated at 100-fold (O), 75-fold (Δ), 50-fold (△), and 30-fold (□) dilution, at constant  $\text{Eu}^{3+}$ -labeled N-IgG concentration to determine the optimal concentration for the assay. B: effect of antibody dilutions on the signal-to-noise ratio

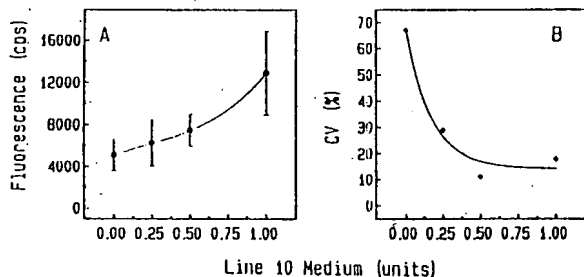


Fig. 4. Sensitivity and intra-assay CV of VPF immunofluorometric assay

Each point in A represents the mean of 10 determinations, and the error bar represents  $\pm 2$  SD. B: intra-assay CV of VPF as a function of VPF dose

centrifugation), little fluorescent signal remained in the supernatant solution. In addition, when guinea pig serum containing platelet-derived growth factor and other growth factors was assayed, no VPF was detected (data not shown).

**Correlation of VPF immunoassay with Miles permeability assay.** We prepared and tested various concentrations of VPF from line 10 medium in both the Miles permeability assay and the VPF immunofluorometric assay. For the Miles assay, the amount of local dye development due to VPF permeability-enhancing activity was quantified by absorbance at 620 nm as described earlier (17). The VPF immunofluorometric assay was more sensitive than the Miles permeability assay; at a dose of 0.35 unit of VPF, the immunoassay gave values that were markedly different from zero (Figure 4A). In contrast, the sensitivity of the Miles permeability assay extended to only  $\sim 10$  units (Figure 6). There was an excellent linear correlation ( $r^2 = 0.94$ ) between the Miles permeability assay and the VPF immunoassay at VPF concentrations  $> 10$  units (Figure 6, inset).

#### Discussion

Immunofluorometric assays involving  $\text{Eu}^{3+}$ -chelate as the label are characterized by low-end sensitivity and

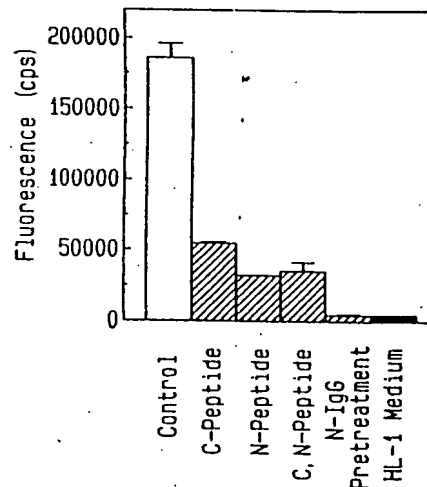


Fig. 5. Specificity of VPF immunofluorometric assay

Line 10 medium was used as the positive control and HL-1 medium was the negative control. C-peptide of VPF (final concentration 80 mg/L) was included during the first incubation with the sample. The N-peptide (final concentration 80 mg/L) was pre-incubated with the  $\text{Eu}^{3+}$ -labeled N-IgG and added during the second incubation. N-IgG pretreatment refers to line 10 medium that has been pre-extracted for VPF by using the N-IgG. Error bars denote  $\pm$  SEM

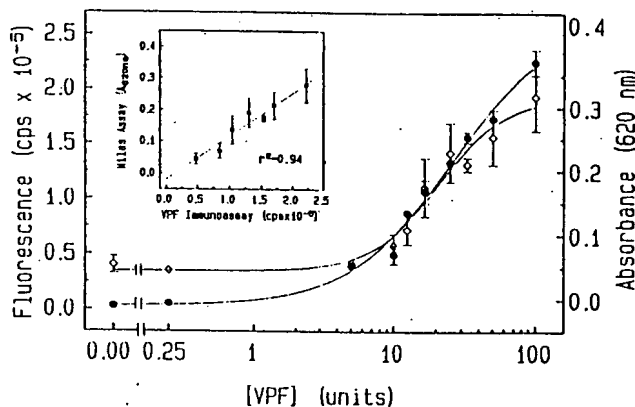


Fig. 6. Correlation of VPF immunoassay and Miles assay

Line 10 conditioned media at various concentrations were prepared for Miles assay (○) and VPF fluoroimmunoassay (●) by diluting with HL-1 medium. Each point and its respective error bar represent the mean  $\pm$  SEM of duplicate determinations. For the Miles assay, two animals were used to generate each point. Inset: correlation of the Miles and the VPF immunoassay at concentrations  $> 10$  units

wide dynamic range. The principles underlying these advantages have been reviewed recently (18). The large Stokes shift of  $\text{Eu}^{3+}$ -chelate and the time-resolved nature of the fluorometric measurements yield a high signal-to-noise ratio. Because of the longer decay time of the  $\text{Eu}^{3+}$ -chelate ( $> 500 \mu\text{s}$ ), readings can be determined between 400 and 800  $\mu\text{s}$  to decrease nonspecific fluorescence, which typically has shorter decay times ( $\sim 0.01 \mu\text{s}$ ). This feature minimizes the endogenous nonspecific fluorescence of various biological specimens, making it especially attractive as a method to measure VPF in both tumor cell culture medium and in biological fluids.

We prepared affinity-purified N- and C-termini antibodies to guinea pig VPF and used the DELFIA approach

to develop a sensitive and specific immunofluorometric assay of VPF. We also demonstrated that our fluoroimmunoassay provides a quantitative measure of VPF in line 10 tumor cell culture medium. Although absolutely pure VPF is unavailable for standardization, we showed good correlation between the VPF immunoassay and the Miles bioassay with line 10 medium, thus confirming that the immunoassay is measuring bioactive VPF. We are confident that the immunoassay is measuring VPF in the physiologically relevant range because the dynamic range correlates well with the dynamic range of the Miles bioassay.

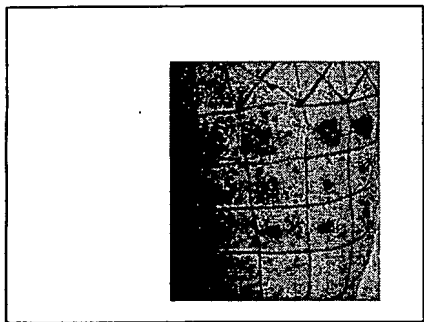
The VPF immunofluorometric assay has a minimal detection limit of 0.35 unit, and is ~30 times more sensitive than the Miles permeability assay. In addition, the immunoassay is much more precise and simpler to perform, is readily automatable, and can measure several specimens rapidly and inexpensively. In a separate study, we used the VPF immunoassay to measure VPF in ascites fluid, serum, and urine to study the potential role of VPF in tumor-elicited ascites fluid accumulation (19). We have also currently adapted the VPF immunofluorometric assay to measure VPF in human fluids to study its potential diagnostic utility in the pathogenesis of tumor metastases and the often accompanying fluid accumulation found in pleural and peritoneal cavities (19).

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**Exhibit**



# Loss of New Chemokine CXCL14 in Tumor Tissue Is Associated with Low Infiltration by Dendritic Cells (DC), while Restoration of Human CXCL14 Expression in Tumor Cells Causes Attraction of DC Both In Vitro and In Vivo<sup>1</sup>

Galina V. Shurin,\* Robert Ferris,<sup>†§</sup> Irina L. Tourkova,\* Lori Perez,\* Anna Lokshin,\* Levent Balkir,\* Bobby Collins,<sup>¶</sup> Gurkamal S. Chatta,<sup>‡</sup> and Michael R. Shurin<sup>2\*†</sup>

Breast and kidney-expressed chemokine (BRAF) CXCL14 is a new CXC chemokine with unknown function and receptor selectivity. The majority of head and neck squamous cell carcinoma (HNSCC) and some cervical squamous cell carcinoma do not express CXCL14 mRNA, as opposed to constitutive expression by normal oral squamous epithelium. In this study, we demonstrate that the loss of CXCL14 in HNSCC cells and at HNSCC primary tumor sites was correlated with low or no attraction of dendritic cell (DC) in vitro, and decreased infiltration of HNSCC mass by DC at the tumor site in vivo. Next, we found that recombinant human CXCL14 and CXCL14-positive HNSCC cell lines induced DC attraction in vitro, whereas CXCL14-negative HNSCC cells did not chemoattract DC. Transduction of CXCL14-negative HNSCC cell lines with the human CXCL14 gene resulted in stimulation of DC attraction in vitro and increased tumor infiltration by DC in vivo in chimeric animal models. Furthermore, evaluating the biologic effect of CXCL14 on DC, we demonstrated that the addition of recombinant human CXCL14 to DC cultures resulted in up-regulation of the expression of DC maturation markers, as well as enhanced proliferation of allogeneic T cells in MLR. Activation of DC with recombinant human CXCL14 was accompanied by up-regulation of NF- $\kappa$ B activity. These data suggest that CXCL14 is a potent chemoattractant and activator of DC and might be involved in DC homing in vivo. *The Journal of Immunology*, 2005, 174: 5490–5498.

**T**he destructive disease head and neck squamous cell carcinoma (HNSCC)<sup>3</sup> annually afflicts 40,000 new persons in the United States (1), and 3,000,000 new cases develop worldwide annually (2, 3). Despite improvements in therapy and diagnosis, the overall survival rate of generally 50% for persons diagnosed with HNSCC has remained practically unchanged over the last two decades (1). For this reason, new therapeutic strategies need to be developed to treat HNSCC and the evaluation of alternative treatment strategies for patients with this malignancy is highly justified. Immunotherapy has a long history, but is only rarely considered as the treatment of choice. However, it seems that increasing efficacy of immunotherapy will make it one of the possible therapeutic options.

Specific active immunotherapy is based on the principle that malignant cells contain immunogenic determinants against which

an antitumor immune response can be induced. Dendritic cells (DC) that acquire Ags from tumor cells are able to induce and regulate specific antitumor immunity. Several clinical trials have been initiated to evaluate the efficacy of DC-based immunotherapies in cancer, including stimulation of endogenous DC (4–6). However, it is still unclear why endogenous DC do not mediate efficient antitumor immunity in cancer patients. Whereas successful immunotherapy requires a functional immune system, a defect in the immune response may contribute to tumor growth. Such defects include active suppression of immune cells including DC by the tumor causing disturbed longevity and cell dysfunction (7, 8). For instance, it has been shown that many tumor cell lines, including melanoma and colon adenocarcinoma can effectively chemoattract DC in vitro, modulate their phenotype, and eventually, severely damage DC mobility (9). From this point of view, recent reports about loss of certain chemokines in several tumors, including HNSCC, initially sound surprising (10–12). However, it is conceivable to hypothesize a new mechanism of tumor escape: loss of certain chemokines by tumor cells results in a low attraction of DC, decreased number of tumor-infiltrating DC and thus inhibited ability of the immune cells to recognize tumor and initiate specific antitumor immune responses. In fact, analysis of phenotype and distribution of immunocompetent cells in oral leukoplakia with different levels of dysplasia revealed that the levels of immune effector cells varied according to the degree of dysplasia (13). Examining distribution of S100<sup>+</sup> DC in the tumor tissues and regional lymph nodes of 60 patients with HNSCC, Deng et al. (14) reported that the S100<sup>+</sup> DC density in tumor tissues was correlated with the tumor histologic grade, and the density of S100<sup>+</sup> DC was significantly higher in regional lymph nodes without tumor than in those with metastases. A similar conclusion was reported after

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<sup>3</sup> Abbreviations used in this paper: HNSCC, head and neck squamous cell carcinoma; SCC, squamous cell carcinoma; DC, dendritic cell.

evaluation of 36 cases of primary HNSCC of the lip mucosa or vermillion border for the correlation between tumor-associated DC density and tumor grade, mitotic rate, diameter, ulceration, depth of invasion, muscle invasion, and metastasis (15). Goldman et al. (16) have determined that survival and recurrence rates for patients with squamous cell carcinoma (SCC) of the tongue correlate with the degree of DC infiltration of the primary tumor or adjacent tongue tissue. Patients who had greater numbers of CD1a<sup>+</sup> DC adjacent to tumor had better survival and decreased recurrence rates. These suggest that the distribution of DC subsets in HNSCC may reflect the degree of tumor immunity induced in the host-bearing HNSCC. Altogether, these suggest a functional role of DC in the immune response to HNSCC. Localized absence of DC might impair mucosal immunologic protection, allow microbial colonization, and enhance carcinogenesis. However, the mechanisms and chemokines responsible for DC homing and accumulation in HNSCC are unknown.

The various members of chemokine are subdivided into four families known as either the CXC, C-C, C, and CX3C, or the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subfamilies, respectively (17). Approximately 50 human chemokines and 20 receptors are currently known. This large number reflects the highly complex traffic pattern of blood leukocytes, including granulocytes, monocytes, lymphocytes, and DC. Accumulating evidence indicates critical regulatory roles for chemokines during the development of metastatic tumors by stimulating angiogenesis and tumor growth. In addition, by regulating immunity, chemokines critically regulate antitumor immune responses and chronic inflammation such as that associated with various neoplasias (18–20).

Breast and kidney-expressed chemokine (BRAK) CXCL14 is a new CXC chemokine with unknown function and receptor selectivity (11, 12, 21). CXCL14 transcripts are highest in human kidney, small intestine, and liver tissues and expressed constitutively by a variety of epithelia including the basal keratinocytes and dermal fibroblasts of skin (21). Importantly, Hromas et al. (12) reported that CXCL14 mRNA was expressed ubiquitously in normal tissues, but absent in a variety of in vitro established tumor cell lines. Moreover, using differential display and in situ mRNA hybridization, Frederick et al. (11) have recently reported that squamous epithelium constitutively express CXCL14, whereas expression in tumors was heterogeneous, with the majority of HNSCC and some cervical SCC showing loss of CXCL14 mRNA. This study demonstrates for the first time up-regulation of CXCL14 mRNA in the inflammatory sites in the tumor microenvironment and lost expression from certain cancers in vivo. The loss of expression in tumors and the presence of CXCL14 in nonmalignant tissues suggest that this chemokine may play a role in host-tumor interactions. It is also possible that down-regulation of the CXCL14 gene expression in tumor cells might be beneficial for tumor growth. However, the role of CXCL14 in the regulation of migration of DC in cancer and their biologic significance has not yet been investigated.

In the present work, we have established new in vitro and in vivo models to address the chemotactic interaction among human DC, CXCL14, and HNSCC tumor cells. We have demonstrated that HNSCC tissues are low in tumor infiltrating DC although DC are present in oral dysplasia lesions. Decreased infiltration of HNSCC by DC was correlated with no or low expression of CXCL14 protein at the tumor site. However, intense CXCL14 staining was observed in oral dysplasia (pre-malignant) lesions. Furthermore, we showed that CXCL14 is a potent DC chemoattractant in vitro and in vivo and DC are recruited to genetically modified CXCL14-expressing HNSCC cells. In addition to being potent DC chemoattractant, CXCL14 also increased functional ac-

tivity of DC, which was associated with increased activity of NF- $\kappa$ B.

## Materials and Methods

### Tumor cell lines and tissues

Human SCC-15, prostate adenocarcinoma LNCaP, and melanoma FemX cell lines were obtained from American Type Culture Collection. The HNSCC PCI-13, PCI-16, and PCI-4B cell lines were prepared from HNSCC tumors (22). Conditioned medium from normal human lymph node cell suspensions served as a positive control. Tumor cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM HEPES (Invitrogen Life Technologies).

The immunohistochemical studies were performed on a variety of formalin-fixed and paraffin-embedded tissue sections, including normal oral tissue (7 blocks), oral epithelial hyperplasia (8 blocks), and oral SCC (8 blocks).

### Mice

Male C.B-17 SCID ( $T^{-/-}$ ,  $B^{-/-}$ ) mice, 6- to 8-wk-old were obtained from Taconic Farms. Animals were maintained in pathogen-free facility under the controlled temperature, humidity, and a 12-h light to dark cycle.

### Immunohistochemistry

Monoclonal Abs recognizing CD83, CD1a (Immunotech), CD11c (DAKO), and S-100 (Sigma-Aldrich) were used for the detection of DC infiltration in formalin-fixed and paraffin-embedded tumor tissue sections. Five-micrometer sections were cut, mounted on positively-precharged slides (Superfrost Plus; Fisher Scientific), and allowed to dry overnight at 56°C to ensure optimal adhesion. The sections were deparaffinized and rehydrated. After endogenous peroxidase quenching (0.3%  $H_2O_2$  in PBS for 30 min), Abs were retrieved by boiling the sections in 1 mM EDTA/NaOH solution, pH 8.0, in a microwave oven for three cycles (5 min each). Appropriately diluted mouse anti-human Abs against CD83 (1/50), CD1a (nondiluted), CD11c (1/100), and S-100 protein (1/1000) were applied to each section. Immunohistochemistry was performed using an avidin-biotin peroxidase technique. Staining was developed with peroxidase and amino-9-ethylcarbazole or diaminobenzidine (Vector Laboratories).

Expression CXCL14 protein in tissue sections and tumor cell lines was determined with anti-CXCL14 mAbs (1/100, overnight incubation; R&D Systems). Staining with normal murine IgG2a was performed as a negative control for CXCL14 stain. Immunohistochemical and immunocytochemical staining was performed using avidin-biotin peroxidase technique described.

### Transduction of HNSCC cell lines with the CXCL14 encoding vector

Frederick et al. (11) have demonstrated expression of CXCL14 in PBMC stimulated with LPS. We isolated total RNA from human PBMC activated with *Escherichia coli* LPS (0.5  $\mu$ g/ml; Sigma-Aldrich) for 6 h using the TriReagent (Molecular Research Center) and according to the supplier's instructions. Up to 2  $\mu$ g of total RNA was reverse-transcribed in a final reaction volume of 25  $\mu$ l containing 2.5  $\mu$ M oligonucleotides, 1 $\times$  reaction buffer (Invitrogen Life Technologies), 0.5 mM each of dNTP (Invitrogen Life Technologies), 10 mM DTT, 1  $\mu$ l of RNase inhibitor (Boehringer Mannheim), and 200 U of Superscript II reverse transcriptase (Invitrogen Life Technologies). For PCR, 4  $\mu$ l of cDNA was amplified in a final volume of 30  $\mu$ l containing 1 $\times$  Taq buffer, 50  $\mu$ M each dNTP, and 2.5 U of Taq polymerase enzyme (Invitrogen Life Technologies). Primers for human CXCL14 were: 5'-CAG GTC GAC ATG AGG CTC CTG GCG GCG GCG and 3'-CGG GGA TCC CTA TTC GTA GAC CCT GCG. PCR amplification was performed at 94°C for 10 min, followed by 35 cycles at 94°C for 1 min, 64°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were resolved by agarose electrophoresis and stained with ethidium bromide. To construct a eukaryotic expression vector, the CXCL14 gene was cloned into pCR3.1 plasmid. The PCR products were purified using QIAEX II gel extraction kit (Qiagen) and cloned into pCR3.1 mammalian expression vector using the Eukaryotic ClonEXpression kit (Invitrogen Life Technologies) according to the manufacturer's instructions.

Human HNSCC primary cell line PCI-16 was transfected with the human CXCL14 gene using Effectene Transfection Reagent (Qiagen) according to the supplier's instructions. Briefly, tumor cells were counted and plated at 70% density in 10-cm petri dish 1 day before the transfection. On the next day, the

medium was removed, the cells were washed in HBSS (Invitrogen Life Technologies), and transfection mixture containing 2  $\mu$ g of DNA was added to the tumor cells. The cells were incubated with a transfection mixture at 37°C for 6 h. After incubation, fresh medium (RPMI 1640, 10% FCS) was added and tumor cells were incubated at 37°C for additional 48 h. Next, transfected tumor cells were split and fresh medium containing 1 mg/ml geneticin (Invitrogen Life Technologies) was added for selection of transfected cells. Culture medium with geneticin was changed twice a week for 2–3 mo. Expression of CXCL14 protein was confirmed by Western blot (recombinant human CXCL14 served as a positive control; RDI) and immunocytochemistry.

### RT-PCR

Analysis of mRNA expression of human chemokines in normal oral mucosa and HNSCC tissues was performed using RT-PCR technique. RNA was extracted from five normal mucosae and five oral SCC specimens, transcribed into cDNA using 200 U of superscript reverse transcriptase, and cDNA was amplified with 2.5 U of *Taq* polymerase using 1.5 pM of the primers specific for MIP-3 $\alpha$ , MIP-3 $\beta$ , CXCL8, and GAPDH. RT-PCR was conducted as described earlier (23).

### Generation of human monocyte-derived DC

CD14-derived DC were generated as described earlier (24). Briefly, PBMC were isolated from buffy coats by Ficoll gradient centrifugation. The PBMC were further plated at  $10^7$  cells/well in 2 ml of AIM-V medium (Invitrogen Life Technologies) in six-well plates. After 1-h incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, nonadherent cells were removed and adherent monocytes were gently washed with warm AIM-V medium. Adherent monocytes were cultured with recombinant human GM-CSF (1000 U/ml; PeproTech) and IL-4 (1000 U/ml; PeproTech) in complete RPMI 1640 medium for 7 days. Maturation of DC was stimulated by additional supplementation with 20 ng/ml TNF- $\alpha$  (PeproTech) on day 6.

### Analysis of DC migration and chemotaxis in vitro and in vivo

Spontaneous and chemokine-induced migration of DC in vitro was assessed in the Transwell system with DC placed in the upper chamber ( $10^6$  cells/ml, 100  $\mu$ l) and chemokines added to the bottom chamber (600  $\mu$ l) in a 4-h migration assay. Cell migration was measured in 48-well Transwell plates (5- $\mu$ m pores; Corning Costar). Recombinant human MIP-1 $\alpha$  (10–20 ng/ml; PeproTech), synthetic chemotactic peptide N-fMLP (0.5–5  $\mu$ g/ml; Sigma-Aldrich) and recombinant human CXCL14 (5–200 ng/ml; RDI) were diluted in RPMI 1640 medium contained 1% FBS (assay medium), and 600- $\mu$ l aliquots were placed in the lower chamber of Transwell plates. Assay medium was used to measure a spontaneous migration of DC. After 4-h incubation at 37°C, the Transwell inserts were removed and cells from the lower chamber were collected. Cells transmigrated through the 5- $\mu$ m pore size membrane were acquired on FACScan (BD Biosciences) for 60 s. Data are reported as mean number of transmigrated cells from triplicate wells.

To test whether human tumor cell lines produce chemokines that attract DC, cell-free conditioned media were collected from FemX, LNCaP, and different HNSCC cell lines. Tumor cells were seeded at  $1 \times 10^6$  in 4 ml of assay medium. Twenty-four hours later, cell-free supernatant was collected and centrifuged. Conditioned medium from normal human lymph node cell suspensions served as a positive control. Tumor-conditioned or control media were placed in the lower chamber of the Transwell plate and migration of DC was assessed as previously described.

Trafficking of DC in vivo was assessed in immunodeficient SCID mice (Taconic Farms) bearing human CXCL14-positive or CXCL14-negative PCI-16 HNSCC. Mice were injected s.c. with  $10^7$  CXCL14-positive or CXCL14-negative HNSCC cells and  $2 \times 10^6$  human DC labeled with fluorescent dye 5-sulfofluorescein diacetate/succinimidyl ester (SFDA/SE, 2.5  $\mu$ M; Molecular Probes) were injected i.v. 1 wk after tumor cells administration. Tumors were harvested 48 h later and tissue sections were analyzed by confocal microscopy and immunohistochemistry with anti-CD1a Abs.

### Flow cytometry

Expression of DC specific markers was determined as described earlier (25) by flow cytometry on a FACSCalibur (BD Biosciences) using the following Abs: CD14-FITC, HLA-DR-PE (BD Biosciences), CD1a-PE, CD40-PE, CD80-PE, CD83-PE, (Immunotech/Coulter), and CD86-FITC (BD Pharmingen). The analyses were done using the CellQuest software (BD Biosciences).

### MLR assay

MLR assays were performed to evaluate the effect of CXCL14 on the ability of human DC to stimulate proliferation of allogeneic T cells. Control and CXCL14-treated (200 ng/ml) DC were added in triplicates in graded doses ( $10^2$ – $10^6$  cells per well) to T cells ( $1 \times 10^5$  per well) in round-bottom 96-well plates. Proliferation of T cells was measured 72 h later by incorporation of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well; DuPont-NEN) added for the last 16 h. Cells were harvested onto GF/C glass fiber filter paper (Whatman) and isotope incorporation was assessed by 1450 MicroBeta TRILUX liquid scintillation counter (Wallac). The counts were expressed as cpm  $\pm$  SEM.

### NF- $\kappa$ B activity assay

Monocyte-derived DC were treated with CXCL14 200 ng/ml for 0–30 min. TNF- $\alpha$  (50 ng/ml, 15 min) served as a well-known activator of NF- $\kappa$ B in DC. Nuclear extract from Jurkat cells was used as an internal control. The effect of CXCL14 on NF- $\kappa$ B activation in DC was determined using a method developed by Active Motif. This method was developed in an ELISA format and uses binding of the active form of NF- $\kappa$ B to immobilized oligonucleotides corresponding to NF- $\kappa$ B nuclear consensus site 5'-GGGACTTTC-3'. The assay was performed according to the manufacturer's specifications.

We additionally quantitated NF- $\kappa$ B in DC by an activity assay recently developed by Marligen Biosciences using a Luminex technology. The assay is based on a specific binding of transcription factors to cognate sequences on labeled probes. Nuclear extracts were incubated with a mixture of PE-conjugate oligonucleotides containing appropriate cognate DNA binding sequences. This mixture was then incubated with a digestion reagent. In the presence of active transcription factors, label remains associated with the probes, whereas it is removed in the absence of transcription factor binding. Finally, the oligonucleotides were captured onto distinctly colored agarose microspheres that allow each of the reactions to be individually scored, and the quantitative signal generated by the label was detected with a Bio-Plex (Bio-Rad) reader. The amount of label remaining correlates with the amount of active transcription factor derived from the nuclear extract. This format allows better sensitivity and dynamic range than does EMSA. Furthermore, quantitative results allow comparisons among treatments. The assays were performed according to manufacturer's protocol.

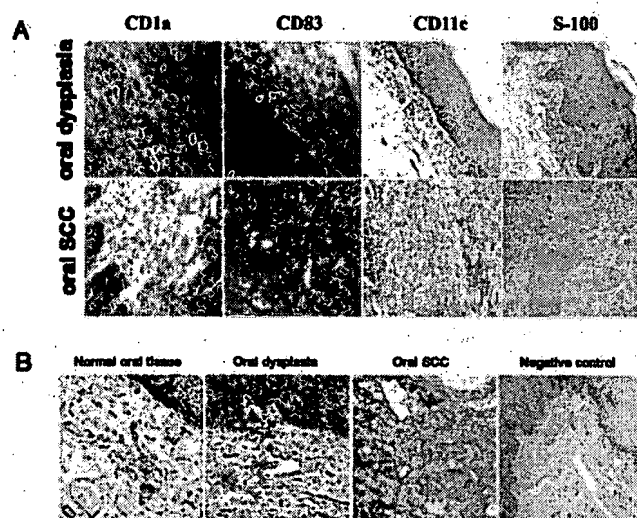
### Statistical analysis

For a single comparison of two groups, the Student *t* test was used after evaluation for normality. If data distribution was not normal, a Mann-Whitney rank sum test was performed. For the comparison of multiple groups, one- or two-way ANOVA was applied. For all statistical analysis, the level of significance was set at a probability of 0.05 to be considered significant. All experiments were repeated at least two or three times. Data are represented as the mean  $\pm$  SEM.

## Results

### Immunohistochemical analysis of human HNSCC tissues for infiltration by DC and expression of CXCL14

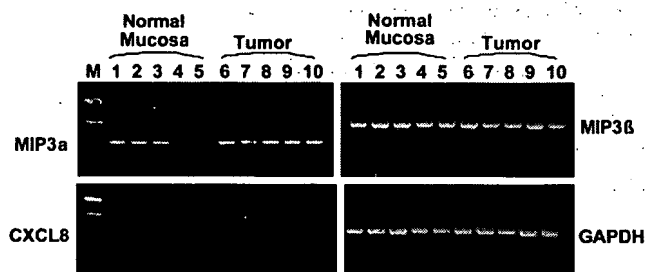
First, we confirmed and expanded the published data concerning the reduction of DC numbers in the tumor mass when compared with nonmalignant tissues (14, 15). We analyzed a variety of paraffin specimens of HNSCC and oral dysplastic lesions for the presence of CD1a, S-100, CD83, and CD11c DC by immunohistochemistry. The biopsy specimens were from different patients diagnosed with mild to moderate dysplasia from oral mucosal sites, including buccal mucosa, lateral tongue, and floor of the mouth. For comparison, specimens were also obtained from patients diagnosed with invasive HNSCC from similar oral mucosal sites. The results of the analysis of multiple oral epithelial hyperplasia, and oral SCC specimens suggest that CD83- and CD11c-positive DC were essentially absent in HNSCC tissues; and the numbers of CD1a- and S-100-positive DC were markedly lower in the tumor tissues than in oral dysplasia lesions (Fig. 1A). These data allowed us to hypothesize that DC migration into the HNSCC tissues might be inhibited compared with their migration to the hyperplastic or premalignant lesions. It is likely that chemokines



**FIGURE 1.** Immunohistochemical evaluation of tumor-infiltrating DC (A) and CXCL14 expression (B) in HNSCC, oral dysplasia, and normal oral tissue specimens. Five-micrometer sections were dried overnight, dewaxed, rehydrated, followed by Ag retrieval with 1 mM EDTA/NaOH solution (see *Materials and Methods*). A, For evaluating tumor-infiltrating DC, the following DC-specific Abs were used: CD83, CD1a, CD11c, and S-100. Secondary Abs were biotinylated with goat anti-mouse. B, For detection of CXCL14 expression in HNSCC tissues anti-CXCL14 Abs or control murine IgG2a were applied to the tissues overnight. Biotinylated horse anti-mouse secondary Abs were added for 30 min. Staining was developed with amino-9-ethylcarbazole and counterstained with hematoxylin. Positive staining is red-brown. The representative immunohistochemical data from the analysis of 10–12 specimens are shown.

are, at least in part, responsible for differential homing of DC in normal and malignant tissues.

To test whether decreased infiltration of HNSCC by DC might be associated with a low expression of chemokines, we have measured expression of DC attracting chemokines in HNSCC and normal mucosa tissues by RT-PCR. Our data revealed similar mRNA expression of MIP-3 $\alpha$  (CCL20) and MIP-3 $\beta$  (CCL19) in HNSCC and normal mucosa (Fig. 2). These chemokines interact with CCR6 and CCR7, expressed on immature and mature DC, respectively. Given that expression of new chemokine CXCL14 mRNA was reported to be lost in different tumors (11, 12), we also examined CXCL14 protein in different human tissues by immunohistochemistry. Because expression of CXCL14 in tissues has been previously determined only by *in situ* hybridization (11, 12), we



**FIGURE 2.** Analysis of chemokine mRNA expression in normal oral mucosa and HNSCC tissues by RT-PCR. mRNA was extracted from normal mucosa and HNSCC specimens, transcribed into cDNA using 200 U of superscript reverse transcriptase, and cDNA was amplified with 2.5 U *Taq* polymerase using 1.5 pM of the primers specific for MIP-3 $\alpha$ , MIP-3 $\beta$ , CXCL8, and GAPDH, as described in *Materials and Methods*. The results of a representative experiment are shown ( $n = 3$ ).

have developed a new immunohistochemical procedure to analyze CXCL14 protein in paraffin-embedded tissues. Fig. 1B demonstrates that both normal oral mucosa tissues ( $n = 7$ ) and oral dysplasia specimens ( $n = 8$ ) were strongly positive for CXCL14, whereas HNSCC tissues ( $n = 8$ ) were low or negative for CXCL14 staining. Thus, these data suggest that CXCL14 protein is lost in human HNSCC, which led us to the hypotheses that low infiltration of HNSCC by DC might be associated with the lost expression of certain chemokines (i.e., CXCL14) and whether CXCL14 is chemoattractive for DC.

#### Chemoattractive properties of CXCL14 and tumor cell lines toward DC

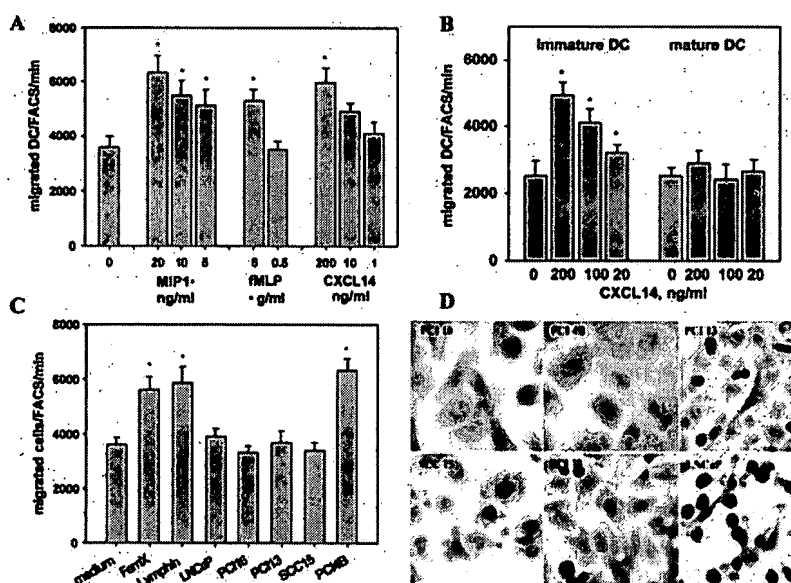
To determine whether DC could be attracted by a CXCL14, we compared its chemotactic activity toward DC with the known DC chemokines. Analysis of DC migration revealed that CXCL14 and two control DC chemokines fMLP, a prototypic bacterial chemotactic stimulus (26) and MIP-1 $\alpha$  (27), all dose-dependently chemoattracted human DC (Fig. 3A). For example, in the presence of 20 ng/ml MIP-1 $\alpha$  migration of immature DC reached  $6360 \pm 650$  cells/min vs  $3620 \pm 380$  cells/min spontaneously transmigrated in control wells ( $p < 0.05$ ). A comparable chemoattraction of DC ( $5960 \pm 568$  cells/min,  $p < 0.05$ ) was also detected in the presence of 200 ng/ml (20 nM) CXCL14 (Fig. 3A). Thus, CXCL14 is a potent DC chemokine with a chemoattractive activity in the nanograms per milliliter range.

Next question was whether CXCL14 chemoattracts both immature and mature DC. Fig. 3B demonstrates that only immature, but not mature DC, are chemoattracted by CXCL14. These data are in agreement with Shellenberger et al. (28) and the general concept that immature DC are attracted to nonlymphoid tissues where a number of potent DC chemokines, including CXCL14, may be ubiquitously expressed.

In the next set of experiments, we tested whether human tumor cell lines, including different HNSCC, prostate adenocarcinoma, and melanoma, might produce chemokines that could attract human DC *in vitro*. Cell-free conditioned media were collected from FemX melanoma, LNCaP prostate adenocarcinoma, and HNSCC cell lines SCC-15, PCI-13, PCI-16, PCI-38, and PCI-4B as described in *Materials and Methods*. Conditioned medium from normal human lymph node cell suspensions served as a positive control. Fig. 3C demonstrates that conditioned media from FemX melanoma cells ( $5620 \pm 483$  vs  $3630 \pm 250$  cells in control,  $p < 0.005$ ), normal lymph node cells ( $5880 \pm 602$  cells,  $p < 0.005$ ), and PCI-4B ( $6340 \pm 436$  cells,  $p < 0.005$ ), but not from LNCaP ( $3920 \pm 286$  cells) and HNSCC lines PCI-13 ( $3720 \pm 405$ ), PCI-16 ( $3320 \pm 241$ ), and SCC-15 ( $3390 \pm 301$ ) ( $p > 0.1$ ), displayed chemoattractive activity toward human DC. Selective attraction of DC by several tumor cell lines raises the question whether it might correlate with the expression of CXCL14.

The next series of experiments focused on evaluating the expression of CXCL14 protein in different human tumor cell lines. Tumor cells were cultured on microscopic slides for 48–72 h, and CXCL14 protein was detected by the immunocytochemical procedure. Human PBMC-derived monocytes stimulated with 0.5  $\mu$ g/ml LPS for 6 h served as a positive control for the expression of CXCL14. Nonstimulated PBMC were used as a negative control. We found that all tested tumor cells, with the exception of PCI4B and FemX, were CXCL14-negative (Fig. 3D). Thus, HNSCC cell line SCC-15 and primary HNSCC cell lines PCI-13, PCI-16, and PCI-38 as well as prostate adenocarcinoma cell line LNCaP express no CXCL14 protein. Interestingly, CXCL14-negative HNSCC cell lines did not attract DC in a chemotaxis assay,





**FIGURE 3.** Analysis of migration of human DC toward different chemokines and tumor cell lines in vitro. DC were generated from CD14<sup>+</sup> monocytes, and DC chemotaxis was assessed in the 5- $\mu$ m pore size Transwell system in 4-h migration assay. The numbers of transmigrated DC were determined by a 60 s FACSscan analysis of triplicate samples. **A**, Comparative analysis of DC migration toward three chemokines revealed a chemoattractive potential of CXCL14. The results of a representative experiment are shown as mean  $\pm$  SEM. Three independent experiments have shown similar results. **B**, Immature (GM-CSF + IL-4, Day 6), but not mature (GM-CSF + IL-4 + TNF- $\alpha$ , Day 8) DC migrate toward CXCL14. The representative results are shown as mean  $\pm$  SEM ( $n = 4$ ). \*,  $p < 0.05$ , one-way ANOVA and  $t$  test. **C**, Differential chemoattraction of human DC to different human tumor cell lines. The results from three independent experiments are shown and presented as the mean  $\pm$  SEM. \*,  $p < 0.05$ , Student  $t$  test. **D**, Immunohistochemical evaluation of CXCL14 expression in tumor cell lines was done as described in *Materials and Methods*. Note the correlation between CXCL14 expression in tumor cell lines (**D**) and their chemoattractive potential toward DC in a migration assay (**C**).

whereas CXCL14-expressing cell lines PCI-4B and FemX demonstrated significant chemoattractive potential for DC in the same assay (Fig. 3D).

#### Migration of DC toward CXCL14-transduced tumors in vitro and in vivo

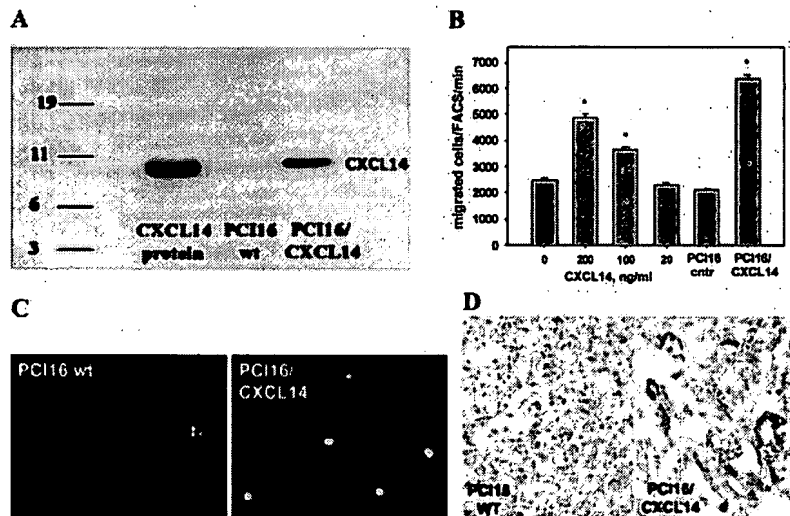
Our results indirectly support the hypothesis that DC are chemoattracted toward CXCL14-producing cells and do not migrate toward at least certain types of tumors that have lost expression of this chemokine. To test this possibility in direct in vitro and in vivo experiments, we have generated a vector encoding human CXCL14, which was used for a stable transduction of human CXCL14-negative HNSCC cell lines. The HNSCC cell line PCI-16 was transfected with the human CXCL14 gene and, after selection, expression of CXCL14 protein was confirmed by Western blot (Fig. 4A). These data suggest that CXCL14-negative tumor cells could be efficiently engineered to produce high levels of CXCL14 protein. Functional activity of synthesized CXCL14 protein in tumor cells was next tested in in vitro and in vivo experiments.

Next we demonstrated that human HNSCC tumor cells transduced with the CXCL14 gene attract significantly higher levels of human DC both in vitro and in vivo. First, cell-free supernatants from CXCL14-negative wild type PCI-16 cultures and PCI-16 cells transduced with CXCL14 were collected and tested for the attraction of human monocyte-derived DC in the chemotaxis assay. Fig. 4B shows that wild type PCI-16 cells did not attract DC ( $2100 \pm 77$  vs  $2500 \pm 105$  cells transmigrated in control wells), whereas CXCL14-expressing PCI-16 cells were chemoattractive for DC ( $6400 \pm 135$  transmigrated cells,  $p < 0.05$ ). Together with the results demonstrating no attraction of DC toward control neo-transduced tumor cells, this suggests that CXCL14-transduced tumor cells release biologically active CXCL14 protein. Second, we

evaluated trafficking of human DC labeled with a fluorescent dye in SCID mice in vivo. Fluorescent-labeled human DC were i.v. injected in immunodeficient SCID mice ( $n = 5$ ) bearing both wild type (or control neo-transduced) and CXCL14-transduced PCI-16 cells for 7 days. Two days later, tumors were harvested and fluorescent cells were examined on 6- $\mu$ m sections immediately by confocal microscopy. The results revealed that infiltration of CXCL14-expressing tumors by labeled DC was significantly higher than in wild type or neo-transduced tumors in all tested mice (Fig. 4C). Similar data were obtained when nonlabeled human DC were i.v. transferred in SCID mice ( $n = 5$ ) bearing PCI-16/wild type (or neo-transduced) and PCI-16/CXCL14 and infiltration of tumors by injected DC was assessed 48 h later by immunohistochemistry. Fig. 4D demonstrates that the levels of accumulation of CD11a<sup>+</sup> human DC in CXCL14-expressing tumors were markedly higher than the number of DC in control tumors. Thus, these results suggest that the recovery of CXCL14 expression in HNSCC cells is associated with increased attraction of DC both in vitro and in vivo.

#### Regulation of DC function by CXCL14

We next tested whether CXCL14, in addition to being a DC chemoattractant, may also increase functional activity of DC. We first examined whether CXCL14 alters phenotype characteristics of DC. Fig. 5 shows that the addition of 200 ng/ml CXCL14 to DC markedly up-regulated expression of CD83, HLA-DR, CD86, and CD80 molecules when compared with CXCL14-untreated DC. For example, the percentage of CD83<sup>+</sup> cells increased from  $8.4 \pm 0.9\%$  in control DC cultures to  $38.0 \pm 2.3\%$  in DC cultures treated with CXCL14 ( $p < 0.01$ ). The same pattern was observed for the expression of CD86 and CD80 molecules on DC (Fig. 5). Interestingly, not only the percentage of DC expressing the specific markers was up-regulated after addition of CXCL14, but also the



**FIGURE 4.** Transduction of CXCL14-negative human HNSCC cell line PCI-16 with the CXCL14 gene resulted in expression of high levels of CXCL14 protein. Primary HNSCC cells PCI-16 were transduced with the human CXCL14 gene as described in *Materials and Methods*. Immunocytochemical analysis of CXCL14-positive cells was assessed by Western Blot (A) as described in *Materials and Methods*. Recombinant human CXCL14 protein served as a positive control. B, CXCL14-transduced HNSCC cells secrete functionally active protein, which exhibits significant chemoattractive potential toward human DC in vitro (B) and in vivo (C and D). PCI-16 cells were transduced with the human CXCL14 gene and after selection with G148 supernatants obtained from wild type (wt) and CXCL14-transduced PCI-16 tumor cells were tested for their ability to attract DC in Transwell-based cell migration assay (B). Medium and CXCL14 served as negative and positive controls, respectively. \*,  $p < 0.05$  vs medium (one-way ANOVA,  $n = 5$ ). C and D, PCI-16 wild type or PCI-16/CXCL14 tumor cells ( $10^7$  cells per mouse) were injected s.c. in SCID mice on day 1. Sulfofluorescein diacetate/succinimidyl ester (SFDA/SE)-labeled or nonlabeled human DC ( $2 \times 10^6$  cells) were injected i.v. on day 7 and all tumors were harvested 48 h later. Tissue sections were analyzed by confocal microscopy (C) or immunohistochemistry with anti-CD1a Abs (red staining) (D) as described in *Materials and Methods*. The results from a representative experiment ( $n = 3$ ) are shown.

levels of CD83, CD86, CD80, and HLA-DR expression on DC were significantly up-regulated (Fig. 5). For instance, the mean fluorescence intensity values for CD83 and HLA-DR markers were increased from  $4.4 \pm 0.5$  in control DC to  $15.9 \pm 1.4$  ( $p < 0.01$ ) on DC treated with CXCL14 and from  $123.6 \pm 5.8$  to  $287.7 \pm 9.9$  ( $p < 0.01$ ), respectively (Fig. 5). Thus, it is conceivable that CXCL14 stimulates maturation of DC.

Further confirmation of the biologic activity of CXCL14 was obtained in the MLR assay using DC generated from different donors with or without the addition of 200 ng/ml CXCL14 (Fig. 6A). Significantly higher induction of allogeneic T cell proliferation by CXCL14-treated DC ( $p < 0.01$ ), as compared with untreated DC, was observed. For instance, at DC to T cell ratio 1:30, uptake of [ $^3$ H]thymidine increased from  $17252 \pm 897$  cpm in control to  $30385 \pm 689$  cpm ( $p < 0.01$ ) in group treated with CXCL14.

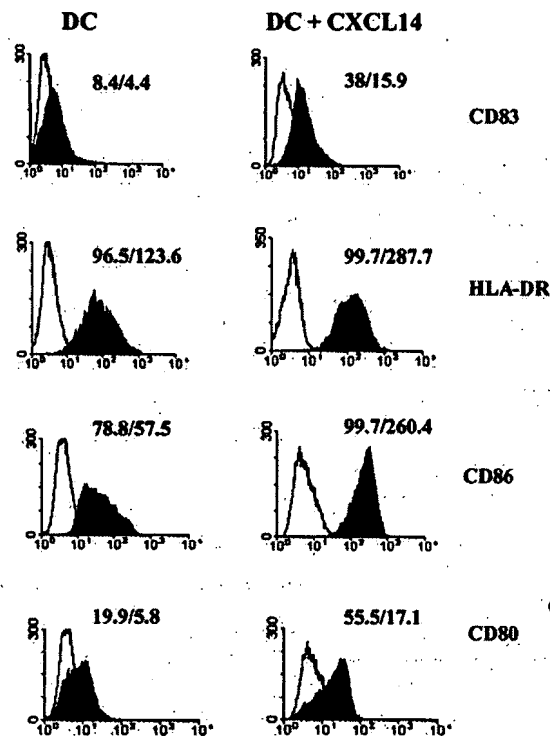
To explore the molecular mechanisms of CXCL14-mediated activation of DC, monocyte-derived DC were treated with CXCL14 (200 ng/ml) and TNF- $\alpha$  (50 ng/ml). The levels of p65 in nuclear extracts were determined using NF- $\kappa$ B Transcription Factor Assay kit. TNF- $\alpha$  served as a well-known activator of NF- $\kappa$ B in DC. Nuclear extract from Jurkat cells was used as internal control. We demonstrated that activation of DC with CXCL14 was accompanied by a significant up-regulation of NF- $\kappa$ B activity in DC up to 200% ( $p < 0.01$ ) (Fig. 6B). Next, these data were confirmed and further explored by using Luminex-based technique for analyzing NF- $\kappa$ B activation (Fig. 6C). The results also demonstrated that CXCL14 is a strong inducer of NF- $\kappa$ B activation in human DC. Interestingly, the kinetic analysis of transcription factor activity revealed that NF- $\kappa$ B activation induced in DC by CXCL14 was delayed compared with TNF- $\alpha$ -induced activation reaching the maximum at 30 min (Fig. 6C).

In summary, these data suggest that CXCL14, in addition to being DC attractant, also increases functional activity of DC.

## Discussion

We have demonstrated that expression of a new DC chemokine, CXCL14, is frequently lost in HNSCC tissues, which was accompanied by a low infiltration of the tumor by DC. We speculate that low levels of HNSCC infiltration by DC may be due to a low or absent expression of CXCL14 in tumor cells. It is well known that homing of leukocytes to the sites of hemopoiesis, Ag priming, immune surveillance, and inflammation largely depends on the presence of chemokines (29). The presence of DC, macrophages, and lymphocytes in solid tumors is regulated by local production of chemokines by tumor and stromal cells. In particular, CC chemokines are the major determinants of macrophage and lymphocyte infiltration in carcinomas of the breast and cervix, sarcomas, and gliomas (30). CCL2 (MCP-1) has been implicated in mediating macrophage infiltration into breast (19) and ovarian cancers (31), whereas CCL5 levels correlate with the extent of CD8 T cell infiltrate in ovarian tumors (20). It is conceivable to speculate that immature DC might be constitutively recruited to CXCL14-expressing tissues. This would allow DC and monocytes to leave the circulation and enter these tissues in the absence of inflammation. On the contrary, the loss of CXCL14 expression in malignant tissues may explain a decreased rate of DC attraction and thus augments efficacy of tumor escape mechanisms.

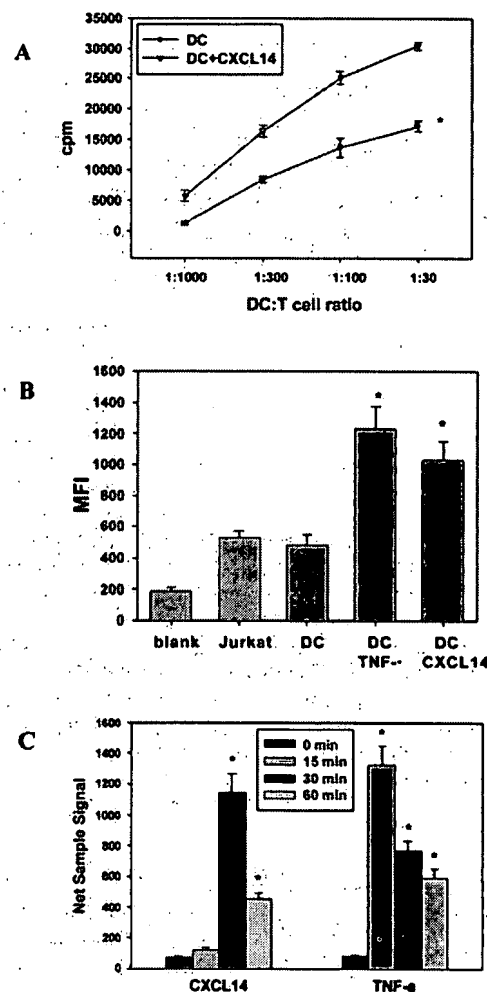
CXCL14 was initially named BRAK because it was identified in human breast and kidney derived cells (12). CXCL14 (KS1, Kcc, BMAC, NJAC, MIP-2 $\gamma$ ) is a chemokine with an as yet unknown function and receptor selectivity (11, 12, 21). The mature sequences of CXCL14 and its murine analog SK1 contain 77 amino acids and are unique with regard to the short N-terminal end of only two amino acids (Ser-Lys), preceding the first of four chemokine-typical Cys residues. The most closely related chemokines, MIP-2 $\alpha$  and MIP-2 $\beta$ , share ~30% amino acid identity with CXCL14. Kurth et al. (32) have recently provided evidence that



**FIGURE 5.** CXCL14 up-regulated DC maturation. Monocyte-derived DC were coincubated with CXCL14 (200 ng/ml, 72 h) and surface expression of CD83, CD80, CD86, and HLA-DR was assessed by FACSscan. Both the percentage and the mean fluorescent intensity (%/MFI) are demonstrated. The results of a representative experiment are shown ( $n = 3$ ).

CXCL14 is not a chemoattractant for peripheral blood T cells, B cells, and NK cells or neutrophils and is selectively chemotactic for monocytes activated by the cyclic AMP-elevating agents PGE<sub>2</sub> and forskolin. The authors proposed that once monocytes enter tissues in response to local inflammation, PGE<sub>2</sub> at the site renders them responsive to the high levels of CXCL14 in these tissues, attracting them to the subepithelial locations where they mature into macrophages. In contrast, others have reported that CXCL14 regulates trafficking of B cells (21), is a potent chemoattractant for neutrophils, and weak or inactive for DC, monocytes, NK cells, and T and B lymphocytes (33). Thus, the data on the biologic role of CXCL14 for chemoattraction of immune cells are controversial.

Our results demonstrate that human recombinant CXCL14 and CXCL14-transduced HNSCC cell line PCI-4B are potent inducers of DC migration in vitro and in vivo, whereas CXCL14-negative HNSCC cell lines and prostate adenocarcinoma cell line LNCaP do not attract DC in a chemotaxis assay. Several laboratories demonstrated that CXCL14 mRNA is constitutively expressed in normal tissues, but absent in a number of tumors (11, 12, 34). The majority of HNSCC and some cervical SCC show loss of CXCL14 mRNA. Analysis of the expression of 20,000 genes in human prostate epithelial cells passaged to senescence revealed the CXCL14 gene among three genes whose expression was uniformly lost in human prostate cancer cell lines and xenografts (34). The loss of expression in tumors and the presence of CXCL14 in nonmalignant tissues suggest that this chemokine may play a role in host-tumor interactions. It is also possible that down-regulation of the CXCL14 gene expression in tumor cells might be beneficial for tumor growth. In agreement, our new data revealed that the growth of CXCL14-transduced murine HNSCC cell line B7E3/6 in syngeneic BALB/c mice was significantly inhibited in comparison with wild type tumors, which was associated with high infiltration



**FIGURE 6.** CXCL14 stimulated APC function of human DC and up-regulates activation of NF- $\kappa$ B. **A**, CXCL14 (200 ng/ml, daily day 3–6) significantly up-regulates Ag-presenting activity of human DC in vitro, as was determined in an allogeneic MLR assay. Data are shown as mean  $\pm$  SEM. \*,  $p < 0.01$ , two-way ANOVA ( $n = 3$ ). **B**, Monocyte-derived DC were treated with CXCL14 (200 ng/ml, 30 min) and p65 was assessed in nuclear extracts as described in *Material and Methods*. TNF- $\alpha$  (50 ng/ml, 15 min) served as well-known activator of NF- $\kappa$ B in DC. Nuclear extract from Jurkat cells was used as an internal control. The levels of p65 in nuclear extracts were determined using NF- $\kappa$ B Transcription Factor Assay kit (Active Motif). Data are expressed as mean  $\pm$  SEM from two independent experiments. \*,  $p < 0.05$  (ANOVA). **C**, NF- $\kappa$ B activity in human DC was determined 0, 15, 30, and 60 min after stimulation with CXCL14 (200 ng/ml) or TNF- $\alpha$  (50 ng/ml) using Luminex-based technique as described in *Materials and Methods*. The results are shown as mean  $\pm$  SEM from two independent experiments. \*,  $p < 0.01$  (ANOVA).

by DC and CD8<sup>+</sup> T cells (G. V. Shurin, R. Ferris, I. L. Tourkova, L. Perez, G. S. Chatta, and M. R. Shurin, manuscript in preparation).

Importantly, a leukocyte and chemokine balance in tumors can be manipulated. When murine tumors are engineered to overexpress certain chemokines, the increased intratumoral infiltrate stimulates antitumor responses. For instance, overexpression of CCL19 (MIP-3 $\beta$ ) mediated rejection of murine breast tumors in an NK cell and CD4 T cell-dependent mechanism (35). CCL21 (6CKine) reduced growth of colon adenocarcinoma in mice using a similar pathway (36). Overproduction of CCL20 (MIP-3 $\alpha$ ) might activate tumor-specific CTLs by attracting DC (37), whereas overproduction of secondary lymphoid tissue chemokine by DC may

enhance T cell recruitment and immune priming to tumor-associated Ags (38). In fact, injection of recombinant secondary lymphoid tissue chemokine in the axillary lymph node region in mice with bilateral multifocal pulmonary adenocarcinomas led to a marked reduction in tumor burden with extensive lymphocytic and DC infiltration of the tumors and enhanced survival (39). Together with clinical evidence demonstrating that infiltration of tumor mass by DC is associated with a better patient survival, these results suggest that regulated induction of DC migration into the tumor site might induce efficient antitumor immune responses. However, there are no data on whether CXC cytokines play a role in attraction of immune cells to the tumor site and inducing antitumor immunity. We have shown, that genetic modification of CXCL14-negative PCI-16 HNSCC cell line with the CXCL14 gene results in stimulation of DC attraction in vitro and increased infiltration of the tumor by DC in vivo. In fact, we have shown on a murine HNSCC model that CXCL14-expressing tumors were highly infiltrated by CD11c<sup>+</sup> DC suggesting their potential role in developing antitumor immune response at the tumor site (G. V. Shurin, R. Ferris, I. L. Tourkova, L. Perez, A. Lokshin, L. Balkir, B. Collins, G. S. Chatta, and M. R. Shurin, manuscript in preparation).

Next, we evaluated the effect of CXCL14 on DC function. It is known that chemokines may regulate cellular adhesion, proliferation, and cell survival (10, 18, 40). Based on the current knowledge of the life cycle of DC, it has been postulated that chemokines can play an important role at several stages of DC development (18). Basal chemokine production and expression at the surface of endothelial cells can mediate DC precursor recruitment into peripheral tissues, which is important for the maintaining DC levels within tissues. Once in the tissue, chemokines, such as MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-3 $\alpha$ , MIP-5, MCP-3, MCP-4, RANTES, TECK, and SDF-1 (41), may participate in differentiation of DC precursors into immature DC that are programmed to pick up and process Ag(s). Upon initiation of an inflammatory response, chemokines that recruit immature DC may be up-regulated, resulting in DC accumulation within the tissue. When DC have matured, they enter tissue-draining lymphatic vessels and migrate to the T cell zones in secondary lymphoid organs under the influence of chemokines produced there, such as MIP-3 $\beta$  and 6CKine. In the T cell zones, DC can produce chemokines that stimulate DC-T cell interaction, thereby enhancing the likelihood of clonal selection (18, 41). Our data show that CXCL14 chemoattracts only immature, but not mature DC, which is in agreement with the concept that nonlymphoid tissue chemokines should attract immature DC. Importantly, we demonstrated that CXCL14 also activated DC through NF- $\kappa$ B-mediated pathways and up-regulated expression of costimulatory molecules on DC as well as enhanced the proliferation of allogeneic T cells in MLR. Thus our results support the hypothesis that CXCL14 might be a novel DC chemokine regulating their homing and activation in nonlymphoid tissues.

## Disclosures

The authors have no financial conflict of interest.

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# B cell- and monocyte-activating chemokine (BMAC), a novel non-ELR $\alpha$ -chemokine

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## Abstract

A novel  $\alpha$ -chemokine, designated KS1, was identified from an EST database of a murine immature keratinocyte cDNA library. The EST has 94% similarity to a recently cloned human gene, BRAK, that has no demonstrated function. Northern analysis of mouse and human genes showed detectable mRNA in brain, intestine, muscle and kidney. Tumour panel blots showed that BRAK was down-regulated in cervical adenocarcinoma and uterine leiomyoma, but was up-regulated in breast invasive ductal carcinoma. KS1 bound specifically to B cells and macrophages, as well as two B cell lines, CESS and A20, and a monocyte line, THP-1. KS1 showed no binding to naive or activated T cells. In addition, KS1 stimulated the chemotaxis of CESS and THP-1 cells but not T cells. The s.c. injection of KS1 creates a mixed inflammatory response in Nude and C3H/HeJ mice. The above data indicates that KS1 and its human homologue represents a novel non-ELR  $\alpha$ -chemokine that may have important roles in trafficking of B cells and monocytes. We propose the name B cell- and monocyte-activating chemokine (BMAC) for this molecule to reflect the described biological functions.

## Introduction

Chemokines are a large family of small peptides that are involved in the trafficking of leukocytes around the body. They consist of proteins between 8 and 12 kDa in size with a number of conserved cysteines that form two disulphide bridges (1–3). The chemokine superfamily is currently classified with respect to the number and position of the first cysteines, CC, CXC, CX<sub>3</sub>C and C. The two main groups are (i) the CXC or  $\alpha$ -chemokines, defined by a single amino acid separating the first two cysteines, and (ii) the CC or  $\beta$ -chemokines, with the first two cysteines being contiguous. The  $\alpha$ -chemokines can be further subdivided into two groups depending on whether they contain a Glu–Leu–Arg (ELR) motif immediately prior to the first cysteine (1). Initially, these molecules were thought to only be involved in stimulating an inflammatory response by promoting chemotaxis of leukocytes from the peripheral blood to sites of inflammation. IL-8 was one of the first chemokines identified and was shown to promote neutrophil migration (4–6). Since then the chemokine family has grown to >50 members (<http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/chemokine.html>) with every leukocyte

population having its own particular subset of chemokines and chemokine receptors. The non-ELR  $\alpha$ -chemokines currently consist of six members whose chemotactic functions are highly diverse (7–13), in contrast to the neutrophil migration-promoting ELR chemokines, and are of great interest for their therapeutic potential in areas other than leukocyte migration. PF-4, IP-10 and Mig have all been shown to have anti-angiogenic properties in a range of tumour models (14–16). SDF-1 $\alpha$  has been shown to competitively block viral entry in HIV strains that uniquely use CXCR4 as their co-receptor for infection (17,18). The potential of this therapeutic approach is supported by the observation that high circulating levels of  $\beta$ -chemokines can confer a degree of immunity on those exposed to HIV (19). Recently, a non-ELR  $\alpha$ -chemokine, BRAK, was identified by screening human EST databases (20). Function has yet to be assigned for this molecule, although it has been postulated to have a role in oncogenesis. We have identified the murine homologue of this gene, the responding cell types for this new chemokine and propose a new name that reflects its biological activity.

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Recombinant human stromal derived factor-1 $\alpha$  and human IL-2 were purchased from PeproTech (Rocky Hill, NJ). The following primary anti-murine antibodies were obtained from PharMingen (San Diego, CA), I-A<sup>k</sup> (A<sup>k</sup>) biotin (clone 11-5.2), CD19-FITC (Clone 1D3), CD4-FITC (clone RM4-5), CD8a-FITC (clone 53-6.7), rat IgG2a-FITC (R35-95) and mouse IgG2b-biotin (clone 49.2). The secondary antibody goat anti-human IgG-phycoerythrin (PE) and streptavidin-PE were purchased from Southern Biotechnology Associates (Birmingham, AL), and the streptavidin-alexa 488 from Molecular Probes (Eugene, OR).

An oligo-d(T)-primed directionally cloned murine immature keratinocyte cDNA library was constructed from poly(A)<sup>+</sup> RNA using a ZAP express cDNA kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. The library was mass excised and colonies randomly selected for sequencing. High-throughput single-pass sequence from the 5' end of the clones was obtained on ABI377 sequencers (Perkin Elmer, Foster City, CA). Novel sequences were analysed using BLAST (21), Prosite (Swiss Institute of Bioinformatics, University of Geneva) and SignalP V1.1 (Center for Biological Sequence Analysis, Technical University of Denmark), and the Phylip package (University of Washington) to define similarities to known gene families or motifs.

The full-length sequence of KS1 was obtained by subcloning and sequence primer walking. The coding region, without the predicted signal sequence, was PCR amplified using KlenTaq polymerase (Clontech, Palo Alto, CA) and KS1 as template, using the following sequences 5'-CATGCCATGGCGTCCAA-GTGTAAGTGTTCCCGGAAGGGG-3' and 5'-CATGCCATGG-CTAATGGTGGTGATGGTGATGTTCTTCGTAGACCCTGCGC-TTCTC-3' as forward and reverse oligonucleotides respectively. The product was purified using a PCR purification kit (Qiagen, Valencia, CA), digested with *Nco*I and ligated into pET16B (Novagen, Madison, WI) to obtain the sequence in frame with the C-terminal (His)<sub>6</sub> tag. In addition to this we cloned the full-length coding region into a eukaryotic expression vector, pIGFc, using 5'-GGAATCCATGAGGCT-CCTGGCGGCCGCGCTGCTC-3' and 5'-ACGGATCCAATTACCTGTTTCTTCGTAGACCCTGCGCTTCTCGTT-3' as forward and reverse primers respectively. PCR products were prepared as above and ligated into pIGFc to obtain the sequence in-frame with human IgG1 Fc present in the vector. All constructs were confirmed by automated sequencing.

KS1 probe was PCR amplified using Taq polymerase (Qiagen) with 5'-ACGCGTCGACATGAGGCTCCTGGCGGC-3' and 5'-TCGTCCAGATCTTTCTTCGTAGACCCTGCGCTT-3' as forward and reverse oligonucleotides respectively. BRAK probe was PCR amplified from human keratinocyte cDNA using Taq polymerase (Qiagen) with 5'-ACGCGTCGACATGAGGCTCCTGGCGGCCGCGCTGCTC-3' and 5'-ATAAGATCTTTCTTCG-

A C-terminal (His)<sub>6</sub> tag fusion protein of KS1 was expressed in BL21(DE3) *Escherichia coli* cells (Novagen). One litre cultures were induced at an OD<sub>600</sub> of 0.5 with 1 mM IPTG and harvested after 3 h. All subsequent procedures were performed on ice. The pellet was re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM PMSF, 10 mM β-mercaptoethanol, 1% NP-40) and sonicated using a Virsonic ultrasonicator (Virtis, Gardiner, NY) fitted with the miniprobe at 20% output for 4×15 s bursts with 15 s intervals. The sonicate was centrifuged in a JA20 rotor at 18,000 r.p.m. for 10 min at 4°C. The resultant pellet was washed twice for 1 h each in lysis buffer containing 0.5% CHAPS and solubilized in 20 mM Tris-HCl, pH 8.0, containing 6 M guanidine-HCl and 0.5 M NaCl. The (His)<sub>6</sub> fusion protein was isolated by chromatography using nickel chelating Sepharose FF resin (0.5 ml column; Pharmacia). After loading, the column was washed sequentially with 20 volumes of binding buffer (6 M urea, 0.5 M NaCl and 20 mM Tris-HCl, pH 8.0), 20 volumes of 0.5% sodium deoxycholate in binding buffer and 20 volumes of binding buffer containing 20 mM imidazole. The protein was eluted with 10 volumes 300 mM imidazole in binding buffer. The eluate was dialysed against binding buffer and re-chromatographed as above. Fusion protein in the eluate was then refolded by dialysis against 1 l of 4 M urea, 20 mM Tris-HCl, pH 7.5, overnight while 1 l of 20 mM Tris-HCl, pH 7.5, was pumped into the dialysis beaker at a rate of 1 ml/min. The refolded protein was finally dialysed against 20 mM Tris-HCl, pH 7.5, containing 10% (w/v) glycerol. Preparations obtained were >95% pure as determined by SDS-PAGE using Fragment Analysis.

**A**

-202  
-200  
-150  
-100  
-50

AGCACCAGC GCCAAGCGCA CCAGGCACCG CGACAGACGG CAGGAGCACC  
CATCCAGCGG CGTACTGGAG CGAGCCGAGC AGAGCAGAGA GAGCGTGTCT  
TGAACCCGAG AACCAAGCCG GCGGCGATCC CCGCGCGCCG GCACGCACAG  
GCCGCGCCG TCCTTGCCTC CTTGCTCCCC ACCGCGCCCG TCAGGCCAGC

1 ATG AGG CTC CTG GCG GCC GCG CTG CTC CTG CTG CTC CTG GCG  
1 M R L L A A A L L L L L L L A

43 CTG TGC GCC TCG CGC GTG GAC GGG TCC AAG TGT AAG TGT TCC  
15 L C A S R V D G S K C K C S

85 CGG AAG GGG CCC AAG ATC CGC TAC AGC GAC GTG AAG AAG CTG  
29 R K G P K I R Y S D V K K L

127 GAA ATG AAG CCA AAG TAC CCA CAC TGC GAG GAG AAG ATG GTT  
43 E M K P K Y P H C E E K M V

167 ATC GTC ACC ACC AAG AGC ATG TCC AGG TAC CGG GGC CAG GAG  
57 I V T T K S M S R Y R G Q E

211 CAC TGC CTG CAC CCT AAG CTG CAG AGC ACC AAA CGC TTC ATC  
71 H C L H P K L Q S T K R F I

253 AAG TGG TAC AAT GCC TGG AAC GAG AAG CGC AGG GTC TAC GAA  
85 K W Y N A W N E K R R V Y E

295 GAA TAG GGTGGACGAT CATGGAAGA AAACTCCAG GCCAGTTGAG AGA  
295 E \*\*\*

344 CTTCAGC AGAGGACTTT GCAGATTAAA ATAAAGCCC TTCTTTCTC ACA  
394 AGCATAA GACAAATTAT ATATTGCTAT GAAGCTCTTC TTACACGGGT CAG  
444 TTTTAC ATTTTATAGC TGCTGTGAA AGGCTTCCAG ATGTGAGATC CAG  
494 CTCGCTT GCGCACCAGA CTTCATTACA AGTGGCTTTT TGCTGGGCGG TTG  
544 GCGGGGG GCGGGGGGAC CTCACGCCCT TCCTTTTAA AATAAGGGGT TTT  
594 GTATTTC TCATATGTC ACCACACATC TGAGCTTTAT AAGCGCTGG GAG  
644 GAACAGT GAGCATGGTT GAGACCGTTC ACAGCACTAC TGCTCCGCTC CAG  
694 GCTTACA AAGCTTCCGC TCAGAGAGCC TGGCGGCTCT GTGCAGCTGC CAC  
744 AGGCTCT CCTGGGCTTA TGAAGGCTCA GAGTTTCACT GTGACTCCAC TGT  
794 GGCCCTT GTTGCAGGGC AATTGGGAGC AGGTCTTCT ACATCTGTGC CTA  
844 GAGGAAC TCAGTCTACT TACCAGAAGG AGCTTCATCC CCACCCACCC CCC  
894 ACCCGCA CCCAGCTCA TTCCCTGTG ACAGCCAGGC AAGTGATCCT TAA  
944 AGGAGCT GGGTCTTTT CTTCGAAACT GAGGGTTTCT GAAAGGTCGG CTG  
994 CTTTGGT AGAAGATGCT TCTGAGCAT CCAAAGTCCC CAGCAGTGTG AGA  
1044 AATGAT TCTCGATGTT CGGGAGGACA AGGGAAGATG CAGGATTAGA TGC  
1094 AGGACAC ACAGCCAGAG CTACACATCC TCTTGGCAAT GGGAGCTCCC CCC  
1144 CCCCAAA GCTTTGTTTC TTTCCTTCAC CCCAACAGAA AGTGCACCTC CCC  
1194 TCAGTGA ATACGCAAA AGCACTGTTC TCTGAGTTAG GATGTTAGGA CGA  
1244 TCTCTGC CCCTGCCCTC TCTGTGTAC ATATTGCCCT CAGTACCCTC CCC  
1294 CCACCCC ATGCCACACA CTGCCCTCA TTAGAGGCGG CACTGTATGG CTG  
1344 TGTATCT GCTATGAAA TGCTGAGACC CCTGAGTCT GCATGCAGGT TTC  
1394 ATGTTCT TTCTAAGATG AAAAGAGAAA GTATATAAAT ATATTGAAG TTC  
1444 CCAAAA AAAAAAAAAA A

**B**

KS1 .....M RLAAA...L LLLLLALCAS RVDSG.....KKKCSRK  
BRK .....M RLPA...L LLLLLALYTA RVDSG.....KKKCSRK  
mCrg-2 .....MNPAAVIFP LILLGLSQT GIPAR.....TVRNCIHD  
mMig .....MKSAVLFLG IIFLEQGVV CTLPAR.....NARCSCSTS  
mSDF-1 .....MVDKVVAVLA LVLAALCISD GKPVSL.....YRCPFRFE  
mBLC .....M RLSTAT...L LLLASCLSP GHGILEAHYT NLKRCRSGVI  
mMIP-2 .....M AP...PTC RLSSAALVLL LLLATNHOAT GAVVAS.....ELRCQCLTL  
mKC .....M IP...ATR SLICAA...L LLLATSLRAT GAPTAN.....ELRCQCLTL  
mLix .....MSLQLRSSAH :PSGSSSPFM RMAPLA.FLL LFTLPQHLAE AAPSSVIAAT ELRCVCLTVT

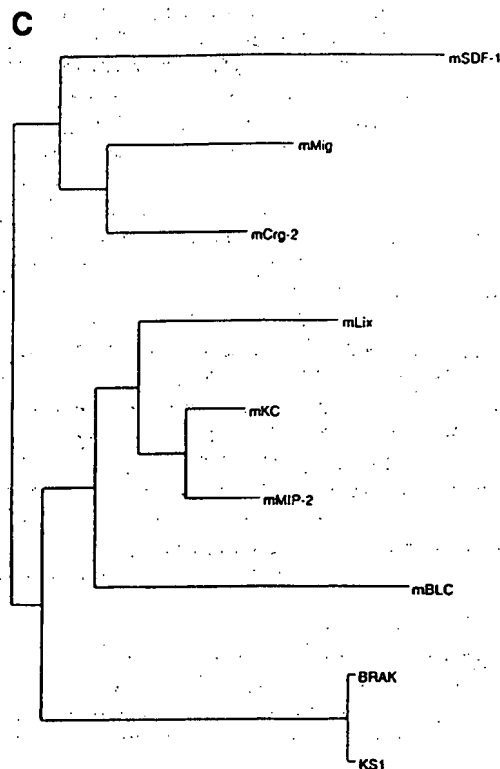
Consensus c c

KS1 PK.IRYSDVK KLEMKPKYPH CEEKVIVIT KMSRYRGGE HCLHPKLQST KRFI...KW  
BRK PK.IRYSDVK KLEMKPKYPH CEEKVIVIT KMSRYRGGE HCLHPKLQST KRFI...KW  
mCrg-2 DGPVVRRAIG KLEIIPASLS CPRVEIATM KR...NGEQ RCLNPESKTI KNLM...KA  
mMig RGTIHYKSLK DLKQFAPSPN CNKTEIATL K...NGEQ TCLDPOSANV KKLMEWEKK  
mSDF-1 SH.IARANVK HLKILN.TPN CALQIVARLK N...NNRQ VCIDPKLKI QEYL...EKA  
mBLC STVVGILNID RIQVTPPGNG CPTEVIVIT K...MKKV ICVNPRAKWL QRLLRHVQSK  
mMIP-2 PR.VDFKNIQ SLVTPPGPH CAGTEVIATL K...GGQK VCLDPEAPLV QKII...QK  
mKC AG.IHLKNIQ SLKVLPSGPH CTOTEVIATL K...NGRE ACLDPEAPLV QKIV...OK  
mLix PK.INPKLIA NLEVIPAGIQ CPTEVIVIAK K...NQRE VCLDPEAPVI KII...OK

Consensus c c

KS1 YNAWNE.KRR VYEE.....  
BRK YNAWNE.KRR VYEE.....  
mCrg-2 FSQKRS.KRA P.....  
mMig INQKKKQKRG KKHQKMKNR KPTKPSKRR SRKT  
mSDF-1 LNKRLKM.....  
mBLC SLSTTPQAPV SKRRAA.....  
mMIP-2 ILNKGK.AN.....  
mKC MLKGVF.K.....  
mLix ILGSDK.KKA KRNALAVERT ASVQ.....

Consensus



**Fig. 1.** (A) The nucleotide sequence of KS1 cDNA is shown along with the deduced amino acid sequence using the single letter code. The 5' untranslated region is indicated by negative numbers. The underlined N-terminal amino acids represent the predicted leader sequence and the stop codon is denoted by '\*\*\*'. The poly-adenylation signal is marked by a double underline. The sequence data is available from GenBank under accession no. AF144754. (B) Comparison of the complete open reading frame of KS1 with its human homologue BRK and with the mouse  $\alpha$ -chemokines mCrg-2, mMig, mSDF-1, mBLC, mMIP-2, mKC and mLiX. An additional five residues are present in KS1 and BRK between cysteine 3 and cysteine 4 that have not previously been described for chemokines. (C) A phylogenetic tree of KS1 was constructed against current murine  $\alpha$ -chemokines using Phylip software version 3.57c, and programs protdist and neighbour joining. The figure represents the degree of divergence between each of the family members. The branch lengths are proportional to the numbers of substitutions, based on the amino acid homology, the level of conservation between the different amino acid residues and the rate of evolution. GenBank accession nos for the sequences are (from top to bottom): L12030, M34815, M86829, U27267, J04596, X53798, AF044196, AF073957 and AF144754.



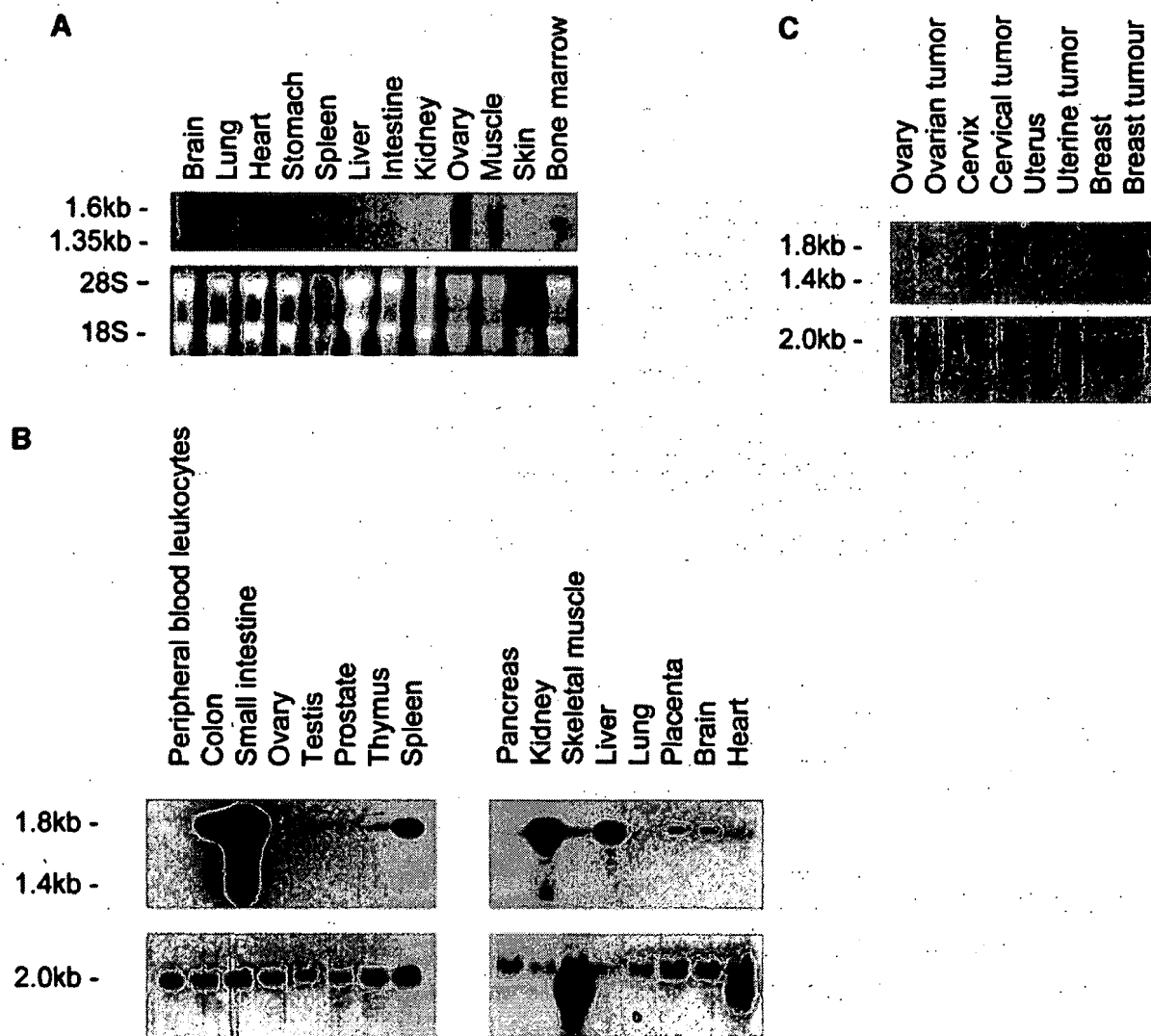
Package (Molecular Dynamics, Sunnyvale, CA). Endotoxin contamination of purified KS1 was determined using a limulus amoebocyte lysate assay kit (Biowhittaker, Walkersville, MD). Endotoxin levels were  $<0.1$  ng/ $\mu$ g of protein. Internal amino acid sequencing was performed on tryptic peptides of KS1 by the Protein Sequencing Unit at the University of Auckland, New Zealand.

An Fc fusion protein was produced by expression in HEK 293 T cells. Using 35  $\mu$ g of KS1pIGFc DNA to transfect  $6 \times 10^6$  cells/flask, 200 ml of KS1 Fc-containing supernatant was produced. The Fc fusion protein was isolated by chromato-

graphy using an Affiprep Protein A resin (0.3 ml column; BioRad, Hercules, CA). After loading, the column was washed with 15 ml of PBS, followed by a 5 ml wash of 50 mM Na citrate, pH 5.0. The protein was then eluted with 6 column volumes of 50 mM Na citrate, pH 2.5, collecting 0.3 ml fractions in tubes containing 60  $\mu$ l of 20 mM Tris-HCl, pH 7.5. Fractions were analyzed by SDS-PAGE and pooled.

#### Cell isolation and culture

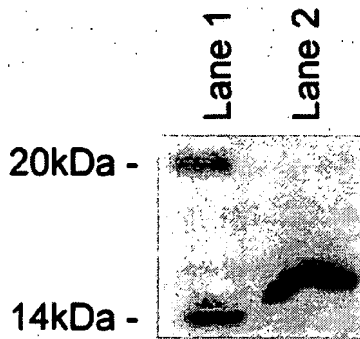
Murine spleens, thymus, peripheral lymph node and bone marrow cells for flow cytometric analysis were obtained from C3H/



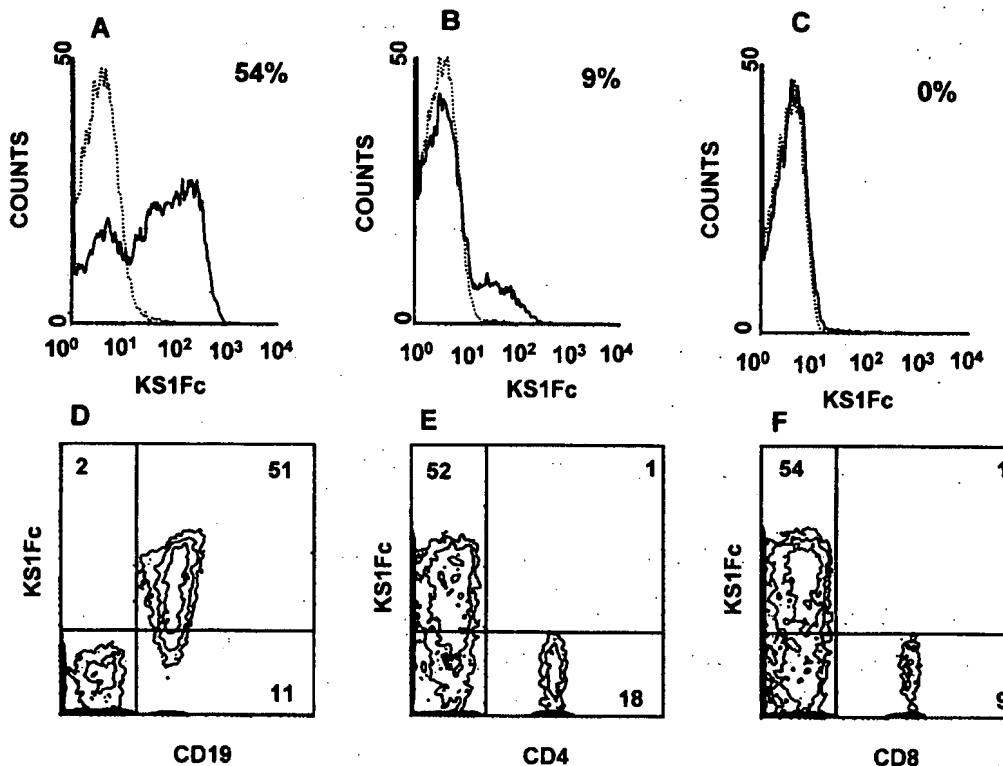
**Fig. 2.** (A). Northern blot analysis of KS1 mRNA in various murine tissues. The upper panel shows the level of expression in these tissues, whereas the lower panel illustrates equal loading of total RNA from these tissues. The position of the 1.35 kb RNA marker is indicated as is the position of 1.6 kb KS1; 28S and 18S ribosomal bands are also indicated. (B) Northern blot of BRAK mRNA in various human tissues as a comparison with murine expression. Human multiple tissue blots were purchased from Clontech. (C) Northern blot analysis of BRAK mRNA in tumor versus normal tissue. Tumor panel blots were purchased from Invitrogen. The northern blot directly compares four different tumors with their respective normal tissue. The upper panels of the human blots shows the level of expression of BRAK, whereas the lower panel demonstrates the level of  $\beta$ -actin expression. The position of the 1.4 kb RNA marker and 1.8 kb BRAK message is indicated.

HeJ mice, erythrocytes were lysed using ACK lysis buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 1 mM  $\text{KHCO}_3$  and 0.1 mM  $\text{Na}_2\text{EDTA}$ ). Peritoneal exudate cells (PEC) were obtained by i.p. lavage from C3H/HeJ mice. In brief, euthanized mice were injected with 2x4 ml volumes of 0.02% EDTA/PBS into the peritoneal cavity using an 18 gauge needle. Cells were then drawn out from the peritoneal

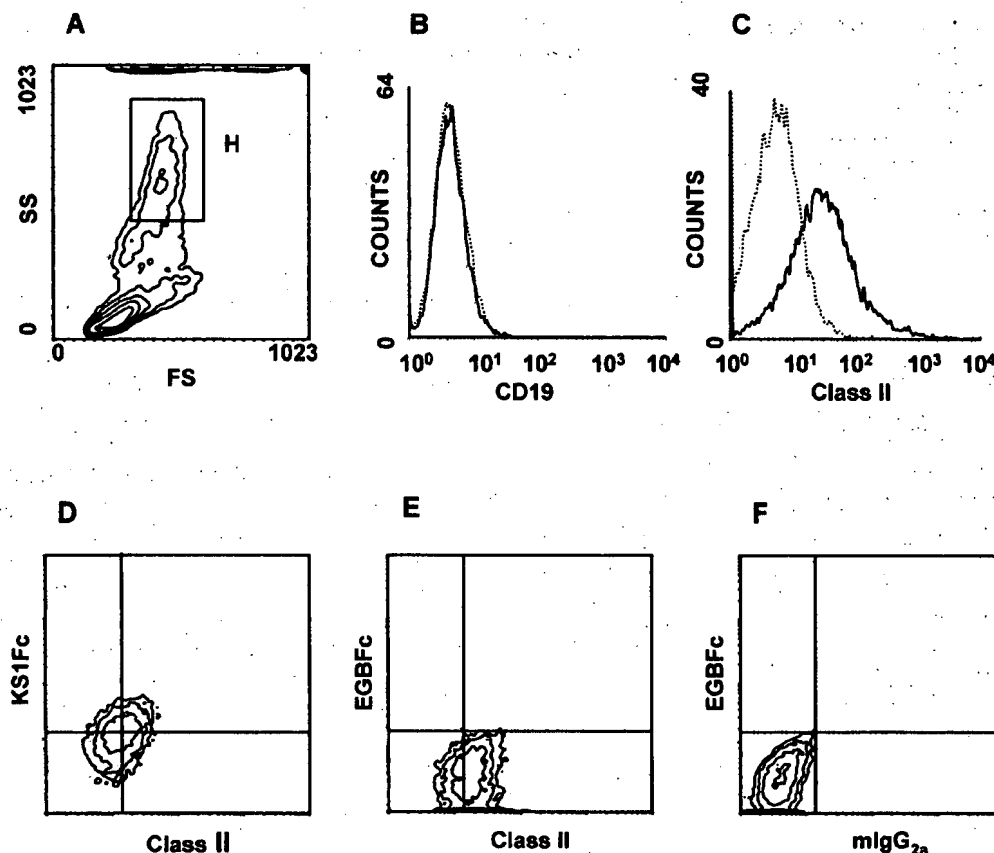
cavity, pelleted and washed in PBS prior to further analysis. Murine IL-2-activated T cells were cultured as described below. Briefly, splenocytes were activated with 2  $\mu\text{g}/\text{ml}$  concanavalin A (Con A) (Sigma, St Louis, MO) in the presence of 5% FBS in DMEM supplemented with 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Life Technologies), 0.77 mM L-asparagine (Sigma), 0.2 mM Arginine (Sigma), 160 mM penicillin G (Sigma), 70 mM dihydrostreptomycin sulfate (Boehringer Mannheim) and 50  $\mu\text{M}$  2-mercaptoethanol, for 3 days followed by addition of recombinant human IL-2 (PeproTech) at 10 ng/ml. Cytokine was added at 3 day intervals for 9–21 days. Peripheral blood mononuclear cells (PBMC) were isolated in heparin (10U/ml) containing tubes from human donors and purified on a Ficol-Hypaque (Pharmacia) gradient by centrifugation at 900  $g$  for 20 min with no brake. PBMC were aspirated from the interface, washed and re-suspended in HBSS, 20 mM HEPES, 0.5% BSA and used directly for assays. Human IL-2-activated T cells were cultured as described below. Briefly, PBMC were activated with 0.1% phytohemagglutinin (PHA) (Gibco/BRL) in the presence of 5% FBS in RPMI supplemented with 2 mM L-glutamine (Sigma), 160 mM penicillin G (Sigma), 70 mM dihydrostreptomycin sulphate (Boehringer Mannheim) and 50  $\mu\text{M}$  2-mercaptoethanol, for 3 days followed by addition of recombinant human IL-2 (PeproTech) at 10 ng/ml. CESS, THP-1 and Jurkat



**Fig. 3.** Analysis of purified  $(\text{His})_6\text{KS1}$  fusion protein by SDS-PAGE. Protein was resolved on a 12% acrylamide denaturing gel and stained with Coomassie blue. Lane 1, mol. wt standards; lane 2, 5  $\mu\text{g}$  of purified  $(\text{His})_6\text{KS1}$  protein.



**Fig. 4.** Flow cytometric analysis of KS1Fc binding to murine splenocytes, peripheral lymph node cells and thymocytes. Cells were labelled with KS1Fc or negative control protein EGbFc and visualized with a two-step staining procedure using goat anti-human-PE. Ten thousand gated events were analysed for each of the experiments. (A) Binding of KS1Fc was detected on murine splenocytes and (B) peripheral lymph node cells as compared with the negative control, EGbFc. (C) Alternatively, KS1Fc showed no binding to thymocytes when compared to negative control. The phenotype of the KS1Fc<sup>+</sup> splenocytes was determined using two-color analysis with the following antibody markers: (D) Murine splenocytes were double positive for KS1Fc and CD19 (D) but not for CD4 (E) or CD8a (F) cells: KS1Fc (solid line), EGbFc (dotted line).



**Fig. 5.** Flow cytometric analysis of KS1Fc binding to monocytes from PEC. Ten thousand gated events were analysed for each of the experiments. Forward (FS) versus side scatter (SS) histogram of PEC (A). All subsequent histograms were gated on region H. Cells from this region were CD19<sup>-</sup> (B) [CD19 (solid line), rlgG2a (dotted line)] and MHC class II<sup>+</sup> (C) [MHC class II (solid line), mlgG2a (dotted line)]. Two-colour analysis shows that MHC class II<sup>+</sup> cells are positive for KS1Fc (D) and not the control protein, EGBFc (E). The matching isotype for MHC class II, mlgG2a, showed no non-specific binding (F).

cells were maintained in complete RPMI as described previously, whereas A20 cells were grown in 5% FBS in DMEM supplemented with 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Life Technologies), 0.77 mM L-asparagine (Sigma), 0.2 mM arginine (Sigma), 160 mM penicillin G (Sigma) and 70 mM dihydrostreptomycin sulphate (Boehringer Mannheim).

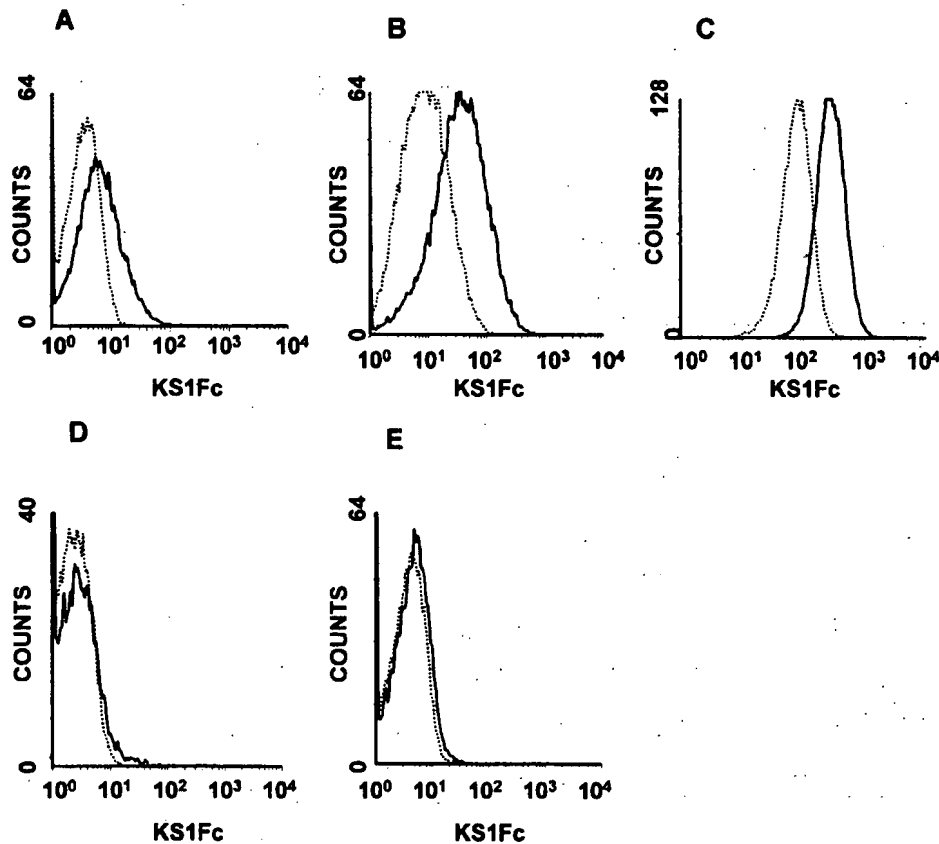
#### Flow cytometric binding studies

Binding of KS1 to cells was tested in the following manner. Cells ( $5 \times 10^5$ ) were resuspended in 3 ml of wash buffer (2% FBS and 0.2% sodium azide in PBS) and pelleted at 4°C, 200 g for 5 min. Ig Fc receptors were blocked with 1% goat serum in wash buffer for 30 min on ice. Cells were washed, pelleted, resuspended in 50 µl of KS1Fc at 10 µg/ml and incubated for 30 min on ice. After incubation the cells were prepared as before and resuspended in 50 µl of goat anti-human IgG-PE at 1 µg/ml and incubated for 30 min on ice. Cells were washed and resuspended in 250 µl of wash buffer containing 40 ng/ml propidium iodide (Sigma) to exclude any dead cells. A purified Fc tagged plant protein (EGBFc) was used, at 10 µg/ml, as a negative control in place of KS1Fc to determine non-specific binding. For two-colour staining, cells were incubated with one of the following antibodies prior to staining with KS1Fc or

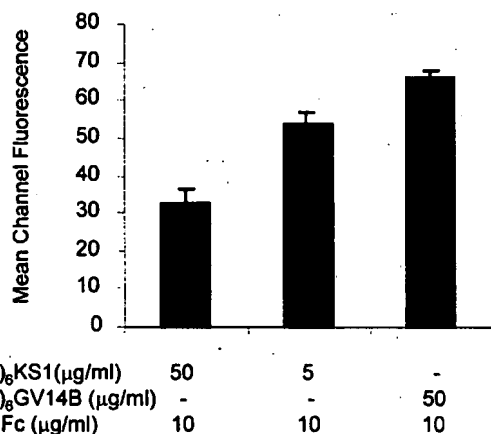
EGBFc, anti-CD4-FITC, anti-CD8a-FITC, anti-CD19-FITC and rat IgG2a-FITC at 10 µg/ml. Biotinylated Ia<sup>k</sup> and its isotype control, mouse IgG2b-biotin, were used to identify MHC class II<sup>+</sup> cells and detected using streptavidin-alexa 488. Ten thousand gated events were analysed on a log scale using a FITC, PE and propidium iodide filter arrangement with peak transmittance at 525, 575 and 675 nm respectively with a bandwidth of 10 nm on an Elite cell sorter (Coulter, Hialeah, FL). To determine KS1Fc binding to human cell lines CESS, THP-1 and Jurkat, and to reduce level of non-specific binding, both KS1Fc and control protein EGBFc were biotinylated using the Sigma biotinylation kit (Sigma BK-101) as described in the manufacturer's protocols. Human cells were labelled with KS1Fc-biotin or EGBFc-biotin as described previously and then detected with streptavidin-PE. Cold competition was performed by adding various concentrations of (His)<sub>6</sub>KS1 at 4°C as a competitor prior to labelling with KS1Fc. An equivalent concentration of (His)<sub>6</sub>GV14B, an identically expressed unrelated bacterial protein, was used as control in competition experiments.

#### Chemotaxis assays

Cell migration in response to KS1 was tested using a 48-well Boyden chamber (Neuro Probe, Cabin John, MD) as described



**Fig. 6.** Flow cytometric analysis of KS1Fc binding to murine and human cell lines. Murine cells were labelled with KS1Fc or negative control protein EGBFc and visualized with a two-step staining procedure using goat anti-human IgG-PE. Human cell lines were labelled with KS1Fc-biotin or negative control EGBFc-biotin and visualized with a two-step staining procedure using streptavidin-PE. Ten thousand gated events were analysed for each of the experiments. Enhanced KS1Fc binding was detected on A20 (A), CESS (B) and THP-1 (C) cells but not on Con A IL-2-activated T cells (D) or Jurkat T cells (E). KS1Fc (solid line), EGBFc (dotted line).

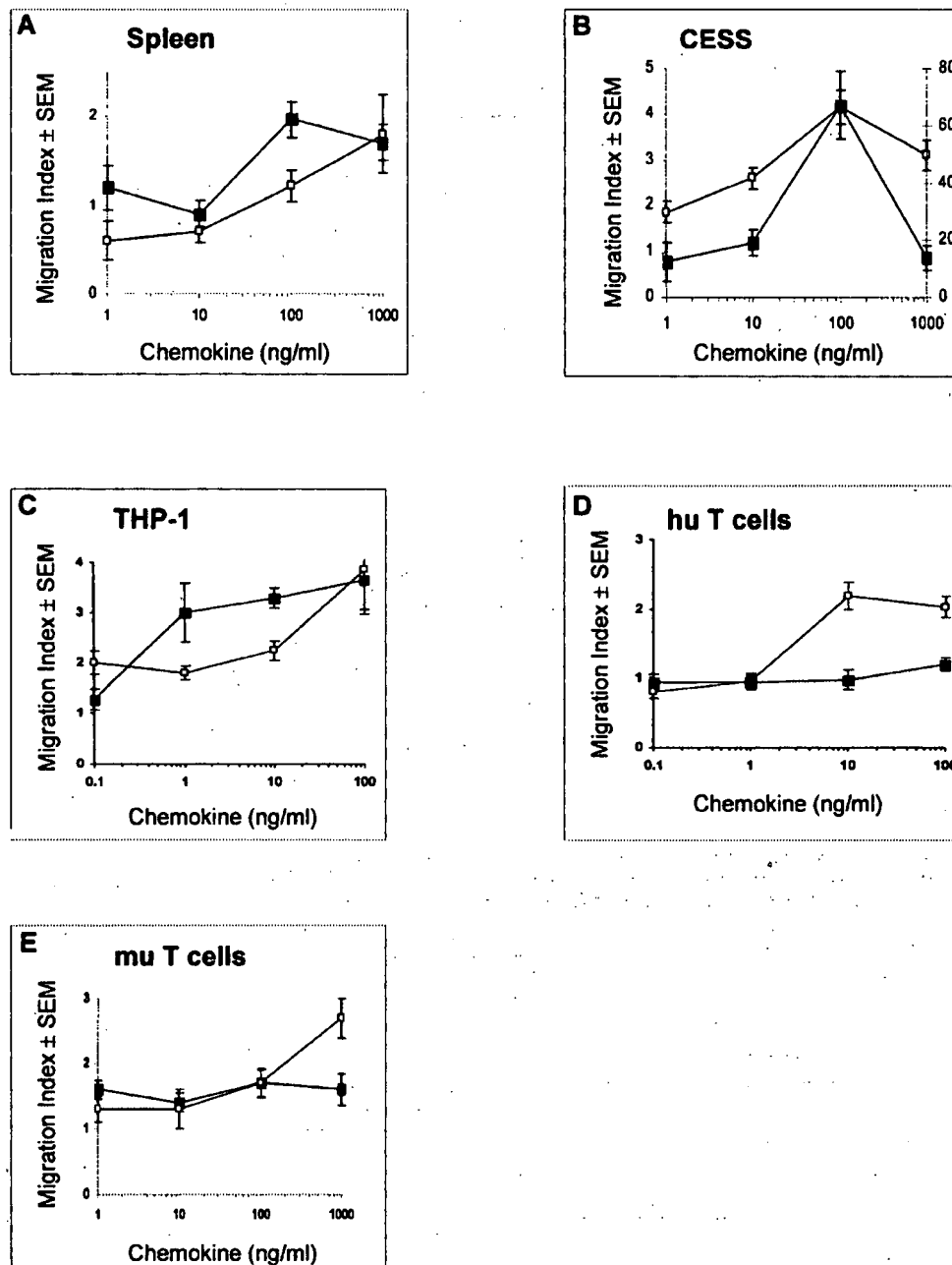


**Fig. 7.** Cold competition of KS1Fc binding with increasing concentrations of (His)<sub>6</sub>KS1. KS1Fc binding on murine splenocytes was inhibited by increasing concentrations of (His)<sub>6</sub>KS1 protein, whereas it was not influenced by the negative control protein, (His)<sub>6</sub>GV14B. Ten thousand gated events were analysed for each experiment. Values are the geometric mean channel fluorescence  $\pm$  SD obtained for duplicate samples and are representative of two individual experiments.

in the manufacturer's protocol. In brief, agonists were diluted in HBSS, 20 mM HEPES, 0.5% BSA and added to the bottom wells of the chemotactic chamber. Cells were resuspended in the same buffer at  $3 \times 10^5$  cells/50  $\mu$ l. Top and bottom wells were separated by a PVP-free polycarbonate filter with a 5  $\mu$ m pore size for CESS and THP-1 cells or 3  $\mu$ m pore size for splenocytes and lymphocytes. Cells were added to the top well and the chamber incubated for 2 h for THP-1 and 4 h for CESS cells, splenocytes and lymphocytes in a 5% CO<sub>2</sub> humidified incubator at 37°C. After incubation the filter was fixed and cells scraped from the upper surface. The filter was then stained with Diff-Quik (Dade Behring Diagnostics, Deerfield, IL) and the number of migrating cells counted in five randomly selected high-power fields. The results are expressed as a migration index defined as: migration index = no. of test migrated cells/no. of control migrated cells. Assays were repeated in triplicate.

#### *In vivo experiments*

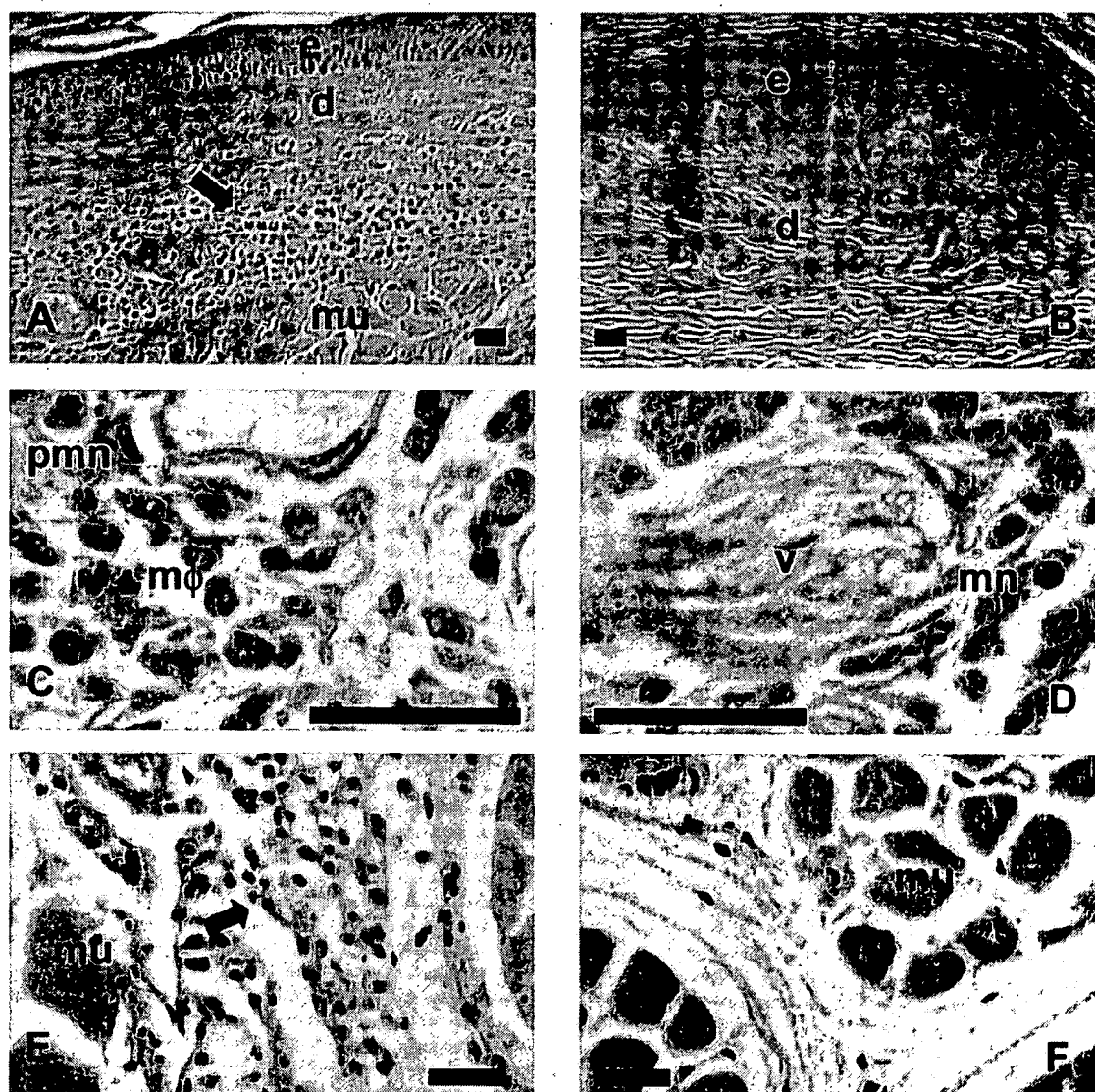
BalbcByJHfh11 *nu/nu* (Nude) and C3H/HeJ inbred mice strains used for all experiments were maintained in house. C-terminal (His)<sub>6</sub>KS1 (20  $\mu$ g) was injected into the left footpads of either Nude or C3H/HeJ mice in triplicate. The right foot of each animal was injected with an equal volume of 20 mM Tris-HCl, pH 7.5.



**Fig. 8.** Chemotactic activities of recombinant KS1. Chemotaxis assays were performed using a 48-well modified Boyden chamber with varying concentrations of KS1 (■) or SDF1α (□). Five randomly selected regions per well were chosen and the number of cells counted under high-power field microscopy. The migration index was calculated by the following formula: migration index = no. of test migrated cells/no. of control migrated cells. (A) Chemotactic activity of murine spleen cells. (B) Chemotactic activity of the B cell line CESS, left y-axis is migration to KS1 whereas right y-axis is migration to SDF1α. (C) Chemotactic activity of the monocyte leukemia cell line THP-1. (D) Chemotactic activity of PHA IL-2-activated T cells and (E) chemotactic activity of Con A IL-2-activated murine T cells. Values are the mean migration index  $\pm$  SEM obtained for triplicate wells and are representative of two individual experiments.

Mice were sacrificed after 18 h, and feet dissected and fixed in 3.7% formal saline. All tissues were sectioned and stained with haematoxylin & eosin. Histology was performed at Agro-

Quality (Auckland, NZ). Photomicrography was performed on a Leica compound microscope and images prepared using Adobe Photoshop.



**Fig. 9.** Inflammatory response of Nude and C3H/HeJ mice upon s.c. injection of KS1. Each group of three mice was injected with (His)<sub>6</sub>KS1 per group. Mice were injected in the left footpad with (His)<sub>6</sub>KS1. Equal volumes of Tris buffer were injected into the right footpads as controls. Feet were biopsied after 18 h and haematoxylin & eosin sections prepared. (A) Nude mice demonstrate a mixed inflammatory response upon injection with KS1, as indicated by an arrow. (B) No inflammation is apparent in the footpad injected with Tris buffer control. (C and D) High-power magnification ( $\times 100$  objective) of cells in Nude mouse inflammation indicates the presence of monocytes, mononuclear cells and polymorphonuclear cells. (E) A mixed inflammatory response was also present in C3H/HeJ mice as denoted by an arrow. (F) No inflammation was detectable in footpads injected with the negative control. Abbreviations: mu, muscle; v, vein; e, epidermis; d, dermis; m $\phi$ , monocyte; mn, mononuclear; and pmn, polymorphonuclear cells. Scale bar = 50  $\mu$ m.

## Results

### Identification of KS1 cDNA sequence

A directionally cloned cDNA library was constructed from immature murine keratinocytes and submitted for high-throughput sequencing. Sequence data from a clone designated KS1 showed 35% identity over 74 amino acids with rat macrophage inflammatory protein (MIP)-2B and 32% identity

over 72 amino acids with its murine homologue. The insert of 1633 bp (Fig. 1A) contained an open reading frame of 300 bp with a 5' untranslated region of 202 bp and a 3' untranslated region of 1161 bp (this sequence is available from GenBank under accession no. AF144754). A poly-adenylation signal of AATAAA is present 19 bp upstream of the poly(A) tail. The predicted mature polypeptide is 77 amino acids in length containing four conserved cysteines with no ELR motif.

The putative signal peptide cleavage site between Gly22 and Ser23 was predicted by the hydrophobicity profile. The full-length sequence was then screened against the EMBL database using the BLAST program, and showed 92.6, 94 and 93.6% identity at the nucleotide level with human EST clones AA643952, AA865643 and HS1301003 respectively. A recently described human  $\alpha$ -chemokine, BRAK, has 94% identity with KS1 at the protein level (20). The alignment of KS1, BRAK and other murine  $\alpha$ -chemokines is shown in Fig. 1B. The phylogenetic relationship between KS1 and other  $\alpha$ -chemokine family members was determined using the Phylip package (Fig. 1C). KS1 and BRAK demonstrate a high degree of divergence from the other  $\alpha$ -chemokines supporting the relatively low homology shown in the multiple alignment.

#### *Tissue expression of KS1 and BRAK*

Tissue distribution of KS1 by Northern hybridization showed high expression in brain, ovary, lung and muscle, with low levels of expression in bone marrow. The transcript size on the Northern blot of 1.6 kb was the same size as the full-length cDNA sequence. BRAK was highly expressed in small intestine, colon and kidney, with moderate to low levels in liver, spleen, thymus, placenta, brain and pancreas. BRAK mRNA could also be detected in skeletal muscle and heart. Expression could not be detected in ovary, testis or prostate. The transcript size of BRAK was ~1.8 kb, which is similar to KS1 (Fig. 2B). As non-ELR  $\alpha$ -chemokines have been implicated as having angiostatic properties, BRAK expression levels were tested in a variety of tumours and compared to normal tissue. BRAK was expressed in normal uterine and cervical tissue, whereas it was completely down-regulated in their respective tumours, uterine leiomyoma and cervical adenocarcinoma (Fig. 2C). Conversely, BRAK was expressed in breast tissue but was up-regulated in breast invasive ductal carcinoma (Fig. 2C).

#### *Recombinant expression of KS1*

Recombinant C-terminal (His)<sub>6</sub>KS1 was a homogenous protein with an apparent molecular mass of 15 kDa (Fig. 3). Internal sequencing of the 15 kDa protein gave the peptide sequence WYNAWNEK, confirming that the observed sequence is identical to that predicted from the cDNA sequence. The isoelectric point was predicted to be 10.26 using DNASIS (Hitachi Software Engineering, Yokohama, Japan). Recombinant KS1Fc, expressed and purified using Protein A-affinity column chromatography, revealed a protein with a molecular mass of 43 kDa corresponding to the predicted size plus the Fc fusion tag (data not shown).

#### *Flow cytometric analysis of KS1 binding*

Fc tagged KS1 (KS1Fc) was used to determine the cell types which express the receptor for this chemokine. KS1Fc bound to 54% of splenocytes and 9% peripheral lymph node cells (Fig. 4A and B). No positive binding was identified in thymocytes (Fig. 4C). Dual labelling experiments with antibodies to cell surface antigens showed that KS1Fc bound B cells in spleen (Fig. 4D) but not CD4 or CD8 T cells (Fig. 4E and F). KS1Fc also bound to the B cells in peripheral lymph node cells but not the T cells (data not shown). The matched

isotype control for CD19, CD8 and CD4, rIgG2a-FITC, showed no positive labelling (data not shown).

Additionally, we screened peritoneal exudate cells (PEC) to determine whether KS1Fc bound monocytes. Forward and side scatter histograms from PEC were used to identify the monocyte population in region H (Fig. 5A). Cells in region H were CD19<sup>-</sup> (Fig. 5B), but were MHC class II<sup>+</sup> (Fig. 5C) indicating that they were monocytes and not B cells. Dual labelling experiments showed that all the cells in region H were double positive for MHC class II and KS1Fc (Fig. 5D). The control protein, EGBFc, showed no binding to the MHC class II<sup>+</sup> cells from region H (Fig. 5E). The matched isotype control for MHC class II, mIgG2a-biotin, showed no positive labelling (Fig. 5F).

As many non-ELR chemokines stimulate activated T cells we analysed KS1Fc binding to Con A-activated splenocytes grown in the presence of 10 ng/ml IL-2 for 9 days. All cells were positive for the activation marker CD69, and consisted of 63% CD4 cells and 37% CD8 cells (data not shown). KS1Fc showed no positive binding to these cells (Fig. 6D). KS1Fc also bound to the murine B cell line, A20 (Fig. 6A), and the human B lymphoblastoid cell line, CESS (Fig. 6B). Additionally, KS1Fc bound to the monocyte leukemia cell line THP-1 (Fig. 6C) but not Jurkat T cells (Fig. 6E). Preliminary analysis identifies B cells and monocytes as responsive cells for KS1. To demonstrate specificity (His)<sub>6</sub>KS1 was used in cold competition with KS1Fc against murine splenocytes. Increasing concentrations of (His)<sub>6</sub>KS1 reduced the level of binding of KS1Fc (Fig. 7), as demonstrated by a decrease in the mean channel fluorescence, to murine splenocytes. An equivalent concentration of a non-specific (His)<sub>6</sub>-tagged protein, GV14B, showed no decrease in mean channel fluorescence when co-incubated with KS1Fc (Fig. 7). The ability of (His)<sub>6</sub>KS1 to competitively inhibit the binding of KS1Fc validates the hypothesis that this reagent bound via the KS1 receptor.

#### *(His)<sub>6</sub>KS1 induces chemotaxis in B cells and monocytes*

Flow cytometric analysis revealed that KS1 specifically bound to B cells and monocytes. We determined whether KS1 could stimulate the chemotaxis of these cells using a modified Boyden chamber. (His)<sub>6</sub>KS1 induced a concentration-dependant migration in murine splenocytes, with optimal activity at 100 ng/ml (Fig. 8A). In addition, (His)<sub>6</sub>KS1 stimulated the migration of the B lymphoblastoid cell line, CESS, and the monocyte line, THP-1 (Fig. 8B and C). However, unlike SDF-1 $\alpha$ , KS1 did not stimulate the migration of either human or murine activated T cells (Fig. 8D and E).

#### *(His)<sub>6</sub>KS1 induces inflammation in vivo*

To determine whether KS1 was active *in vivo* and whether T cells are required for an inflammatory response we injected Nude mice s.c. with (His)<sub>6</sub>KS1. Histological examination of mouse footpads injected s.c. with (His)<sub>6</sub>KS1 showed a leukocyte infiltrate (Fig. 9A). The inflammation was of a mixed phenotype with evidence of mononuclear cells and polymorphonuclear cells (Fig. 9C and D). No obvious inflammation was apparent in the feet of mice injected with Tris, the buffer excipient (Fig. 9B). To confirm that this inflammation was due to (His)<sub>6</sub>KS1 and not endotoxin we repeated the experiment

in LPS-insensitive C3H/HeJ mice. (His)<sub>6</sub>KS1-injected footpads from these mice showed a similar inflammatory response to the Nude mice (Fig. 9E) with the buffer excipient-injected footpads having no marked inflammation (Fig. 9F).

## Discussion

We have identified some of the biological activities of a novel non-ELR  $\alpha$ -chemokine, KS1, and described its tissue distribution. The cDNA was similar to a recently cloned human gene called BRAK (20). Homology of KS1 to BRAK was 94% at the protein level, indicating it as the murine homologue of this gene. To date no known function has been described for BRAK. KS1 and BRAK appear to be distant relatives of the non-ELR  $\alpha$ -chemokines as was shown by their phylogenetic relationship. There are five additional residues (KS1, SMSRY and BRAK, SVSRY), between cysteines 3 and 4 of the conserved cysteines, which is not consistent with the predicted Prosite motif for  $\alpha$ -chemokines. The predicted N-terminus of the mature protein upstream of the first cysteine has only two residues in contrast to other non-ELR  $\alpha$ -chemokines. Furthermore, KS1 has a lysine in place of an arginine immediately prior to the first cysteine. The conservation of a highly basic residue, typically arginine, prior to the first conserved cysteine has been postulated as a requirement for binding to the receptor (1). The amino acid substitutions between KS1 and BRAK in the mature peptide are conservative, indicating that these differences are likely to be insignificant.

KS1 tissue distribution in mouse and BRAK in human is unusual for  $\alpha$ -chemokines in that it is highly expressed in normal non-lymphoid tissues. Although expression levels are different between mouse and human, KS1 and BRAK are expressed in brain and muscle. Differences between mouse and human expression profiles have been described for other chemokines (23–25), and are thought to reflect pathological changes of the particular donor. In contrast to the reported expression profile for BRAK (20), we found BRAK was expressed at higher levels in small intestine, colon and kidney. Additionally, the predominant band ran at 1.8 kb rather than 2.5 kb as reported earlier (20), raising the possibility of splice variance. Alternative splicing has previously been described as a property of some chemokines, for example LARC/MIP3 $\alpha$  (23).

As non-ELR  $\alpha$ -chemokines have been shown to have angiostatic function (15,16,26,27), we investigated BRAK expression in normal versus tumour tissue. The blot revealed that BRAK is expressed in non-malignant breast, uterine and cervical tissues. With good expression in human breast and kidney it is not surprising that BRAK was identified from breast and kidney EST. From the tumour expression data we saw two patterns emerge, either BRAK was up-regulated in breast invasive ductal carcinoma or, in the case of uterine leiomyoma and cervical adenocarcinoma, BRAK mRNA was undetectable. The reason for the disparate trends in expression levels from the different tumours is unclear at this stage and may be related to differences in cancer pathology. Furthermore, these biopsies were likely derived from a single patient and may not reflect the majority of cases. Nevertheless, it would

be of interest to determine whether BRAK added directly to tumour models of these cancers could alter malignancy.

KS1 had a predicted size of 9.4 kDa; however, the purified (His)<sub>6</sub>KS1 protein had an apparent size of 15 kDa which could not be accounted for by the additional histidine residues. This discrepancy between the predicted and apparent size has previously been reported for a number of chemokines (28,29), and is thought to be due to the highly basic nature of these proteins.

The high degree of homology between KS1 and BRAK suggested that they would be active on both mouse and human cell types. This was demonstrated by flow cytometric analysis which showed that KS1 binds directly to mouse and human B cells and monocytes. Furthermore, KS1 induced chemotaxis on both mouse and human cells. This phenomenon of rodent chemokines stimulating human cells has been previously described for a number of different chemokines (30–32). We clearly defined B cells and monocytes, and not T cells, as target cells for KS1 by binding studies. The only other non-ELR  $\alpha$ -chemokine to bind to these cells is SDF-1 $\alpha$ ; however, SDF-1 $\alpha$  also binds to T cells (17,18,33). We were able to confirm that KS1 stimulates B cells and monocytes but not T cells in migration assays using a range of different cell types. In the case of splenocytes and THP-1 cells, SDF-1 $\alpha$  and KS1 stimulated equivalent levels of migration; however, CESS cells were 15-fold more responsive to SDF1 $\alpha$  than KS1.

As the *in vitro* data indicated that B cells and monocytes respond to KS1, we tested its inflammatory properties by injecting Nude mice with the protein. Mice injected s.c. with (His)<sub>6</sub>KS1 showed a mixed inflammatory response. As Nude mice have no T cells this supported the *in vitro* data that (His)<sub>6</sub>KS1 promotes extravasation of cells other than T cells. As seen with the Nude mice, C3H/HeJ also had an inflammatory response to (His)<sub>6</sub>KS1, demonstrating that the response in the Nude mice was (His)<sub>6</sub>KS1 specific and not due to endotoxin. Although we have demonstrated the ability of KS1 to stimulate chemotaxis of B cells and monocytes, we do not rule out the possibility that other haemopoietic or non-haemopoietic cells might respond to KS1.

The majority of non-ELR  $\alpha$ -chemokines have been shown to be chemotactic for activated T cells; however, KS1 did not cause the migration of these cells. Therefore, this raises the question of which receptor does KS1 utilize? There are currently only three known chemokine receptors that bind non-ELR  $\alpha$ -chemokines: CXCR3, the receptor for I-TAC (12), Mig and IP-10 (34); CXCR4, the receptor for SDF-1 $\alpha$  (17,18); and CXCR5, the receptor for BCA-1 (13). As we have shown that KS1 does not stimulate T cells it is unlikely that it is binding via CXCR4. Furthermore, it is unlikely to bind via CXCR3, a receptor on activated T cells, as we can demonstrate no activity on Con A IL-2-activated T cells. This then leaves CXCR5; however, this receptor has only been demonstrated on B cells and not monocytes. Therefore, the likelihood of KS1 acting via a novel receptor merits further investigation.

The biological function of a novel chemokine, initially identified as KS1, is described. KS1 has a broad expression in non-lymphoid tissue, altered expression levels in tumours and a role in trafficking of B cells and monocytes. Therefore, we propose the name B cell- and monocyte-activating chemokine



(BMAC) for this molecule to reflect its described biological functions

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### Abbreviations

BMAC	B cell- and monocyte-activating chemokine
Con.A	concanavalin A
ELR	Glu-Leu-Arg
MIP	macrophage inflammatory protein
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PEC	peritoneal exudate cells
PHA	phytohaemagglutinin

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## Cutaneous response to recombinant interleukin 2 in human immunodeficiency virus 1-seropositive individuals

(human immunodeficiency virus 1 infection/cutaneous anergy/ $\gamma$  interferon)

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**ABSTRACT** We report that 11 human immunodeficiency virus 1 (HIV-1)-seropositive patients, including three AIDS patients, were able to generate a cellular immune response to the intradermal injection of low doses (2–10  $\mu$ g) of recombinant interleukin 2 (rIL-2). A dose-dependent zone of induration appeared at the site of injection, peaked at 24 hr, and was accompanied by the local accumulation of T cells, monocytes, and Langerhans cells. Despite the reductions in the CD4<sup>+</sup> T-cell counts in the peripheral blood of most patients, CD4<sup>+</sup> T cells could still be mobilized with rIL-2 injections into the skin. The total number of immigrant cells was equivalent to those in HIV-1-seronegative patients, although the CD4<sup>+</sup>/CD8<sup>+</sup> ratio of the dermal population was reduced. In response to rIL-2, major histocompatibility complex (MHC) class II antigen was expressed on the surface of keratinocytes, Langerhans cells, lymphocytes, and macrophages. In addition, the  $\gamma$  interferon (IFN- $\gamma$ )-induced protein IP-10 rapidly appeared in dermal inflammatory cells and keratinocytes. A majority of HIV-1-seropositive patients demonstrated low or absent responses to common skin-test antigens. Those with positive zones of induration were often defective in the cellular expression of the IFN- $\gamma$ -induced MHC class II antigen. The simultaneous administration of rIL-2 and soluble antigen at widely separated cutaneous sites led to an enhancement of skin-test antigen reactivity in seropositive patients. The results suggest that local administration of rIL-2 to seropositive patients may act systemically, stimulating cellular immunity to recall antigens, and thus may be of potential benefit in the defense against opportunistic pathogens encountered in HIV-1 infection.

Acquired immunodeficiency syndrome (AIDS), an endstage manifestation of human immunodeficiency virus 1 (HIV-1) infection, is characterized by the progressive loss of CD4<sup>+</sup> T cells (1) and the subsequent defective secretion of lymphokines, including interleukin 2 (IL-2) (2) and  $\gamma$  interferon (IFN- $\gamma$ ) (3). This results in impaired mononuclear phagocyte activation and predisposes the patients to a variety of intracellular infections. Asymptomatic HIV-1-seropositive individuals with mild CD4<sup>+</sup> T-cell deficiencies commonly exhibit cutaneous anergy to skin-test antigens (4) and fail to generate a lymphocyte proliferative response upon stimulation with recall antigens *in vitro* (5). The mechanisms contributing to these events are poorly understood.

We have shown (6) that the intradermal administration of low-dose human recombinant IL-2 (rIL-2) can lead to a delayed-type hypersensitivity (DTH) response that is quantitatively and qualitatively similar to that generated by soluble antigens such as purified protein derivative (PPD) of tuberculin. The cutaneous reaction is accompanied by enhanced proliferation of circulating T cells, the generation of cytotoxic T cells, and the disposal of *Mycobacterium leprae*

(7). We reasoned that rIL-2 may also enhance the cutaneous reactivity of poorly responsive individuals with HIV-1 infection. We now report a comparative study of the effects of local rIL-2 administration in a group of HIV-1-seropositive and -seronegative individuals. We demonstrate that rIL-2 can induce a delayed, cell-mediated immune response in the skin of asymptomatic seropositives and those with AIDS. In addition, rIL-2 can enhance the cutaneous reactivity of seropositive individuals to common skin-test antigens.

### METHODS

After receiving approval from the Rockefeller University Hospital Institutional Review Board, 21 HIV-1-seropositive individuals who are participants in a longitudinal study (8) and 11 HIV-1-seronegative individuals agreed to receive intradermal injections of rIL-2 and/or skin-test antigens. All procedures were carefully explained, and signed consent forms were obtained before the study was initiated. The clinical profile of each participant is listed in Table 1. All seropositive individuals had been skin-tested by us more than 1 year ago and had peripheral blood CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios < 1.0. There was no significant difference in the mean CD4<sup>+</sup> T-cell count among those who received rIL-2 and antigen ( $465 \pm 140$  cells per  $\mu$ l) and those who received antigen alone ( $346 \pm 179$  cells per  $\mu$ l).

**rIL-2 Administration.** rIL-2 (Proleukin, Cetus;  $18 \times 10^6$  international units per mg) was reconstituted in 1.2 ml of sterile water and diluted in sterile 5% dextrose to achieve a final concentration of 1, 2, 5, or 10  $\mu$ g per 100  $\mu$ l. Using a tuberculin syringe with a 27-gauge needle, we injected each individual intradermally on the right side of the back in an area of normal-appearing skin with 1, 2, and 5  $\mu$ g (in descending order). In selected individuals, we chose to inject the diluent or 10  $\mu$ g alone.

**Skin-Test Antigen Administration.** On the left side of the back in descending order, we injected intradermally 5 tuberculin units of PPD (Connaught Laboratories), intermediate strength *Candida* antigen (Hollister-Stier, Elkart, IN), and intermediate strength *Trichophyton* antigen (Hollister-Stier, IN) in 100- $\mu$ l volumes. In some individuals both skin-test antigens and rIL-2 were administered at the same time. These individuals were injected with rIL-2 on the right side of the back and antigens on the left side of the back.

**Measurement of Response.** The diameter of erythema and induration at the site of injection was measured daily with a micrometer. Responses were recorded as negative when there was no erythema or induration. Participants were questioned about local or systemic side effects.

Abbreviations: HIV-1, human immunodeficiency virus 1; IL-2, interleukin 2; IFN- $\gamma$ ,  $\gamma$  interferon; MHC, major histocompatibility complex; LC, Langerhans cells; PPD, purified protein derivative. \*To whom reprint requests should be addressed at: Box 280, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

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Table 1. Clinical profile of HIV-1-seropositive and -seronegative individuals under study

Patient	HIV antibody in serum	Clinical status	Antiviral therapy	CD4 <sup>+</sup> /CD8 <sup>+</sup> (ratio)
1	+	ASX	—	352/1056 (0.3)
2	+	ASX	—	456/988 (0.5)
3	+	ARC	AZT, ACV	189/639 (0.3)
4	+	ASX	—	399/1600 (0.3)
5	+	ASX	—	627/994 (0.6)
6	+	AIDS	—	533/1165 (0.5)
7	+	ASX	—	606/707 (0.9)
8	+	ARC	—	380/450 (0.8)
9	+	ASX	—	675/1535 (0.4)
10	+	ASX	—	441/885 (0.5)
11	+	ASX	—	355/1933 (0.2)
12	+	AIDS	AZT	312/1306 (0.2)
13	+	ASX	AZT	633/1336 (0.5)
14	+	ASX	—	395/687 (0.6)
15	+	ASX	AZT, ACV	556/996 (0.6)
16	+	ARC	AZT, ACV	76/1215 (0.1)
17	+	AIDS	AZT, ACV	36/472 (0.1)
18	+	ASX	AZT, ACV	283/742 (0.4)
19	+	ASX	AZT	479/1059 (0.5)
20	+	ARC	—	338/857 (0.4)
21	+	AIDS	—	70/297 (0.2)
22	—	ASX	—	ND
23	—	ASX	—	1480/640 (2.3)
24	—	ASX	—	740/440 (1.6)
25	—	ASX	—	650/540 (1.2)
26	—	ASX	—	1573/470 (3.3)
27	—	ASX	—	872/340 (2.5)
28	—	ASX	—	726/1022 (0.7)
29	—	ASX	—	ND
30	—	ASX	—	970/330 (3.0)
31	—	ASX	—	ND
32	—	ASX	—	ND

ASX, asymptomatic; ARC, AIDS-related complex; AZT, azidothymidine; ACV, acyclovir; ND, not determined.

\*Cells per microliter of blood.

**Skin Biopsy and Analysis.** Punch biopsies (3 mm) were taken from the indurated sites primarily on day 2 and/or day 5 after injection. The specimens were processed by paraformaldehyde-lysine-periodate fixation (9) and stored in 25% sucrose/5% (vol/vol) glycerol until sectioned in a cryostat at -20°C. Immunocytochemistry was performed as described (6). Mouse monoclonal antibodies (mAbs) were used to distinguish specific mononuclear cell types: Leu-2a, Leu-3a, and Leu-4 (anti-CD8, anti-CD4, and anti-CD3 T cells, respectively) and Leu-M5 (anti-CD11c monocyte/macrophage) from Becton Dickinson; OKT6 [anti-CD1 Langerhans cells (LC)] from Ortho Diagnostics; and 9.3F10 [mAb against major histocompatibility complex (MHC) class II antigen] from our laboratory. Biotinylated horse anti-mouse immunoglobulin (Vector Laboratories) was used as the secondary antibody reagent. Rabbit antibody against IP-10 induced by IFN- $\gamma$  (obtained from J. Ravetch, Memorial Sloan-Kettering Cancer Center, NY) followed by biotinylated goat anti-rabbit IgG antibodies were used to identify the IFN- $\gamma$ -inducible protein, IP-10 (10). The sections were evaluated by light microscopy, and enumeration of positive-staining cells was performed at  $\times 40$  magnification. Photomicrographs were taken with a Nikon Microphot.

**Peripheral Blood CD4<sup>+</sup>/CD8<sup>+</sup> T-Cell Subset Analysis.** Venipuncture was performed prior to the administration of rIL-2 and/or skin test antigens and, in selected patients, after 1, 2, and 4 weeks of injection for routine complete blood cell counts and CD4<sup>+</sup>/CD8<sup>+</sup> T-subset cell analysis.

**Statistical Analysis.** A paired comparison and Student's *t* test were used to determine significance ( $P < 0.01$ ).

## RESULTS

**Clinical Response to Intradermal rIL-2.** The administration of rIL-2 by the intradermal route led to the migration of circulating cells into the injection site and resulted in a zone of erythema and induration, similar to our previous findings in lepromatous leprosy patients (6). A typical skin response after the injection of 1, 2, and 5  $\mu$ g of rIL-2 is shown in Fig. 1. The gross cutaneous changes exhibited by HIV-1-seropositive patients (Table 1, patients 1-10 and 17) were equivalent to those exhibited by seronegative controls (Table 1, patients 22-28). No local reactions occurred at the site of excipient injection.

**Kinetics and dose-response analyses of rIL-2 injection** seen in HIV-1-seropositive and -seronegative patients are shown in Fig. 2. A dose-response correlation following 1-, 2-, and 5- $\mu$ g injections of rIL-2 was observed. Induration at the injection site was maximal 24 hr after injection and persisted for at least 7 days. Neither parameter of the intradermal response was significantly different in the two groups. Reactions 24 hr after injection were occasionally accompanied by local warmth and pruritus but did not lead to systemic complaints. Peripheral blood CD4<sup>+</sup> T cells and the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios of injected seropositive patients were not significantly different among those tested 1, 2, and 4 weeks after injection.

**Immunohistological Response to rIL-2.** Punch biopsies from the center of reactional sites resulting from the intradermal injection of 2  $\mu$ g and 5  $\mu$ g of rIL-2 were obtained 2 days and 5 days postinjection, respectively. In both HIV-1-seropositive and -seronegative patients, the mononuclear leukocyte inflammatory infiltrate recruited into the injected site occupied ~10-35% of the dermis. Immunostaining indicated that there was a similar distribution of T cells, monocytes/macrophages, and LC in the dermal infiltrate. T cells were distributed closely in the perivascular regions and diffusely near the epidermis (Fig. 3 A and B). Appreciable numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found in the skin of both seronegative and seropositive individuals, but the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios of the dermal infiltrate were quite different. Seropositive patients had mean ratios of <1.0, whereas seronegative patients had mean ratios of 2.0 (Table 2). These ratios were a reflection of the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios of the

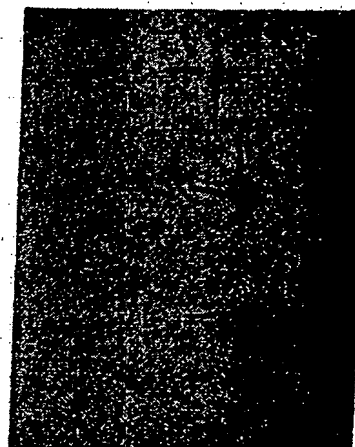


FIG. 1. Response to intradermal injection of rIL-2. Photograph of an individual 24 hr after injection of 1, 2, and 5  $\mu$ g of rIL-2 down the right side of the back. Induration is noted in a dose-dependent fashion at the three rIL-2 infection sites.

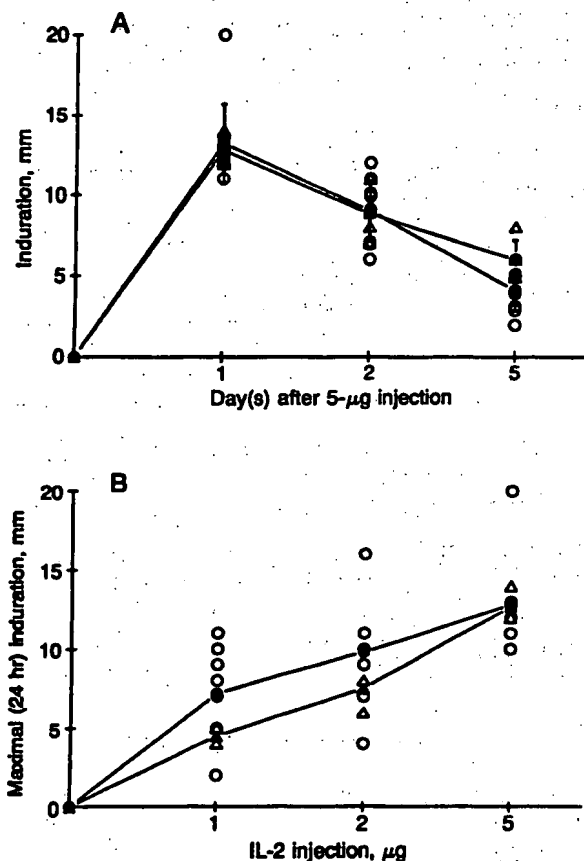


FIG. 2. Response to rIL-2, measured as the diameter of induration at the injection site, in HIV-1-seropositive (○) and -seronegative (Δ) individuals; ● and ▲ represent mean values. (A) Response was maximal at 24 hr after injection. (B) Induration became maximal 24 hr after injection, and the degree of induration was dose-dependent. There was no significant difference in response among the two groups tested.

peripheral blood compartment. With the passage of time, 2–5 days postinjection, the CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios of seropositive patients decreased progressively (Table 2). It is uncertain whether this represented the preferential loss of CD4<sup>+</sup> T cells and/or the continued recruitment of the CD8<sup>+</sup> T-cell subset. However, it is clear that seropositive patients with or without AIDS were able to mobilize significant numbers of CD4<sup>+</sup> T cells into the skin in response to rIL-2.

The number of LC per ×40 field was not significantly different in the uninjected skin of HIV-1-seropositive and -seronegative individuals (Table 2). After rIL-2 injection, the number of CD1<sup>+</sup> LC was slightly higher in the thickened epidermis and in the upper dermis (Fig. 4), but similar numbers were present in both groups. In both cases ~30–50% of the LC strongly expressed MHC class II antigen. The injection of rIL-2 modified the phenotype of keratinocytes overlying the injection sites. In both seropositive and seronegative individuals, both MHC class II antigen and IP-10 were expressed on the keratinocytes (Fig. 4 and Table 2). Both effects are known to result from the presence of IFN-γ, and it is likely that rIL-2 initiates the local production of IFN-γ in both patient groups.

**Clinical and Immunohistological Response to Skin-Test Antigens.** HIV-1-seropositive and -seronegative individuals were skin-tested with three common antigens. The seropositive group showed the following positive responses (any measurable induration): 85% to *Candida* antigen, 20% to *Trichophyton* antigen, and 5% to tuberculin PPD. The sero-

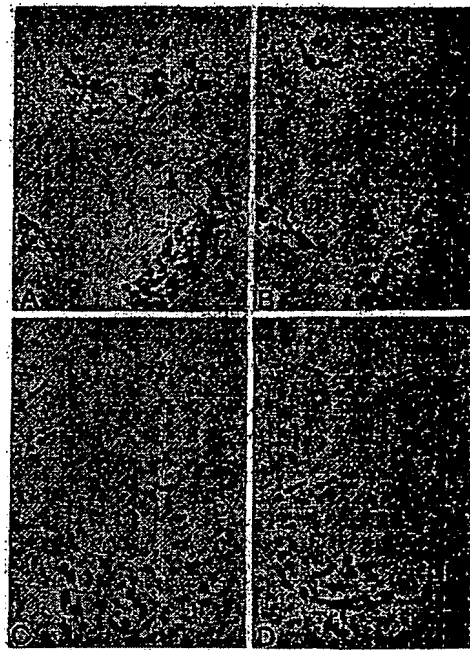


FIG. 3. Photomicrographs depicting immunoperoxidase staining of skin biopsies from HIV-1-seropositive patient 7 taken 2 days after a 2-μg rIL-2 injection. (A) Dermal distribution of CD3<sup>+</sup> T cells staining with Leu-4. (B) CD8<sup>+</sup> T cells staining with Leu-2a in the dermis and epidermis. (C) CD4<sup>+</sup> T cells staining with Leu-3a in the dermis and epidermis (arrows). (D) CD8<sup>+</sup> T cells staining with Leu-2a in the dermis and epidermis (arrows). (A and B, ×100; C and D, ×250.)

negative group demonstrated an overall greater positive response rate, with 100% to *Candida*, 50% to *Trichophyton*, and 33% to tuberculin PPD. The time course of local induration was maximal at 48–72 hr and was similar in both groups. However, there were significantly larger zones of induration in the seronegative group than in the seropositive group ( $P < 0.001$ ).

Biopsies taken 2 and 5 days after antigen injection demonstrated a mononuclear cell infiltrate, the extent of which was in proportion to the zones of induration and occupied from 15–50% of the dermis. The infiltrate contained T cells, monocytes/macrophages, and LC, the number and distribution of which were similar to those induced with rIL-2. The CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios of dermal T cells were again much higher in seronegative individuals (Table 2).

Unlike the response to rIL-2, epidermal keratinocytes from the majority of HIV-1-seropositive patients failed to demonstrate surface membrane MHC class II antigens upon immunostaining. In contrast, all of the seronegative patients expressed MHC class II staining 5 days after antigen injection. No differences were noted in the percentage of CD1<sup>+</sup> LC, which expressed MHC class II antigen in seropositive or seronegative patients. Whereas the expression of MHC class II antigen on keratinocytes was subnormal in seropositive patients, IP-10 was present in all biopsies examined (Fig. 4).

**Enhancement of Skin-Test Responsiveness by rIL-2.** Our findings show that through the selective loss of CD4<sup>+</sup> T cells, HIV-1 infection results in depression of the cell-mediated immune response to skin-test antigens. As shown in the previous section, rIL-2 can by itself evoke cell-mediated immune response in the skin of HIV-1-infected individuals. Therefore we evaluated the role of rIL-2 in enhancing skin-test reactivity in HIV-1-seropositive patients. For this purpose, rIL-2 was administered simultaneously with skin-test antigens, but in widely separated sites, in seropositive indi-

Table 2. Cellular response to rIL-2 and skin-test antigen determined by immunostaining of skin biopsies from test sites

Biopsies analyzed, no.	HIV antibody in serum	Injection on day 0	Day of biopsy	Dermis CD4 <sup>+</sup> /CD8 <sup>+</sup> T-cell ratio*	Epidermis			
					LC, no. per ×40 field <sup>†</sup>		Keratinocytes <sup>‡</sup>	
					CD1 <sup>+</sup>	Class II <sup>+</sup>	Class II <sup>+</sup>	IP-10 <sup>+</sup>
rIL-2								
7	+	2 μg	2	0.77 ± 0.19 (a)	20 ± 11	12 ± 2	5/7	7/7
7	+	5 μg	5	0.48 ± 0.11 (b)	22 ± 20	10 ± 6	5+2/7	6+1/7
3	—	5 μg	5	2.00 ± 0.24 (c)	15 ± 1	5 ± 1	3/3	3/3
7	+	Antigen	2-3	1.25 ± 0.49 (d)	15 ± 6	6 ± 1	1/7	7/7
4	—	Antigen	2-3	2.02 ± 0.30 (e)	12 ± 7	9 ± 4	1+1/4	4/4
3	+	Antigen	5	0.85 ± 0.25	16 ± 1	8 ± 1	1/3	3/3
3	—	Antigen	5	6.84 ± 2.30	13 ± 2	9 ± 1	3/3	3/3
7	+	Antigen + rIL-2 <sup>§</sup>	5	0.87 ± 0.27	22 ± 14	7 ± 3	1/7	7/7
4	+	None	—	ND	9 ± 5	4 ± 2	0/4	0/4
4	—	None	—	ND	10 ± 7	5 ± 1	0/4	1/4

\*Mean values from all biopsies analyzed ( $P < 0.01$ : a vs. b; b vs. c; d vs. e). ND, not determined, as normal uninjected skin does not contain significant numbers of T cells.

<sup>†</sup>A minimum of 10 ×40 fields was counted.

<sup>‡</sup>No. of biopsies positive for induced MHC class II antigen or IP-10 on keratinocytes over the total no. of biopsies evaluated. Italicized numbers indicated patchy staining, with only small areas of keratinocytes staining.

<sup>§</sup>Patients also received 5 μg of rIL-2 at another site.

viduals. The results are shown in Table 3. In the case of tuberculin PPD, an antigen to which only 1 of 20 patients demonstrated any previous sensitization, rIL-2 was without effect. A similar result was obtained with *Trichophyton* antigen. However, the situation was quite different with *Candida* antigen, to which most patients were minimally or moderately responsive. Here, there was a significant enhancement in skin-test reactivity in the majority of seropositive patients 1–10 ( $P < 0.005$ ). The enhancement of skin-test reactivity was based upon comparisons with prior tests done as long as 1 year previously. Patients not receiving rIL-2 failed to show appreciable increments in skin-test reactivity compared with prior testing (patients 11–20), unless antiviral therapy had been initiated.

rIL-2 at a distant site induced more extensive areas of induration as a result of increased cellular infiltration in response to *Candida* injection. The cellular distribution was similar to that noted with rIL-2 and antigen alone (Table 2).

We conclude that low-dose rIL-2 can accentuate the cell-mediated immune response to skin-test antigens to which the patient had been sensitized.

## DISCUSSION

Patients infected with HIV-1, with depressed levels of CD4<sup>+</sup> T cells and low CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios, can still mount a vigorous cell-mediated immune response in the skin after the administration of rIL-2. The response encompasses the generation of a variety of signals leading to the effective homing, transmigration, and accumulation of peripheral blood mononuclear cells into the dermis. The process leads to release of IFN-γ, either through activation of T cells or through stimulation of natural killer cells. This is expressed as the IFN-γ-induced changes in the dermis and epidermis. We suspect that by supplying rIL-2, thereby bypassing the need for large numbers of CD4<sup>+</sup> helper T cells, we initiate and maintain the sequelae of the cell-mediated reaction in a

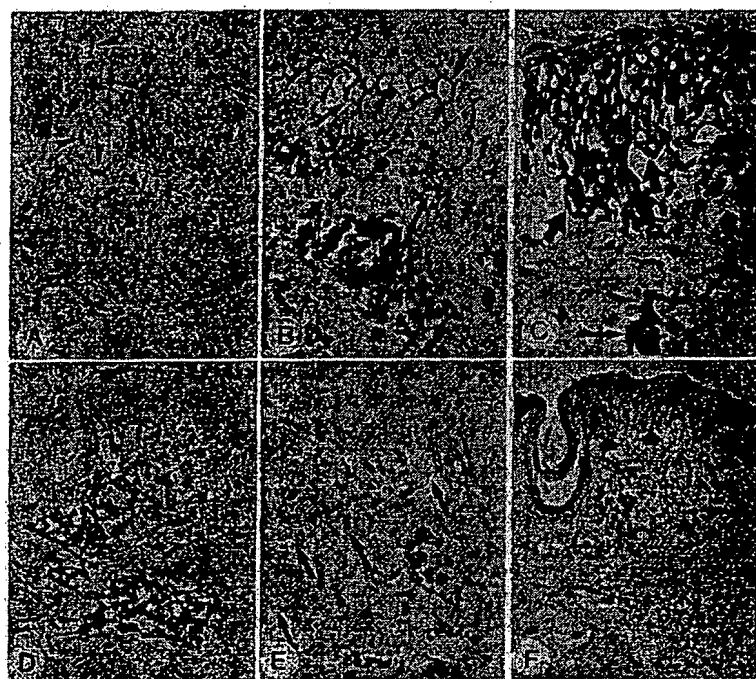


FIG. 4. MHC class II antigen and IFN-γ-induced peptide (IP-10) expression in the dermis (mononuclear cells) and epidermis (keratinocytes and LC) in HIV-1-seropositive patients. (A) MHC class II staining of cells in the dermis and epidermis of a skin biopsy taken 2 days after a 2-μg rIL-2 injection. The basal keratinocytes have been induced to express class II antigen on their surface (arrows). LC also stained in both the epidermis and upper dermis (arrowheads). The inflammatory cells of the dermis are also Ia<sup>+</sup>. (B) High magnification of A. Surface staining of the keratinocytes is shown (arrows). (C) IP-10 expression in the keratinocytes and dermal inflammatory cells 2 days after a 2-μg rIL-2 injection (large arrows). All keratinocytes and some of the dermal inflammatory cells are highly positive (small arrows). (D) Lack of MHC class II expression on the keratinocytes (arrows) of a biopsy taken 5 days after *Candida* antigen injection (13-mm induration at 48 hr). The mononuclear cells of the dermis are positive for MHC class II antigen. (E) High magnification of D. The keratinocytes of the epidermis are clearly not staining (arrows). (F) IP-10 expression in the keratinocytes (arrows) and some of the inflammatory cells of the dermis 5 days after *Candida* antigen injection. (A, D, and F, ×100; B, C, and E, ×250.) Biopsy samples were from seropositive patient 7 (A–C) and seropositive patient 4 (D–F).

Table 3. The effect of rIL-2 on the delayed-type hypersensitivity response to common antigens in HIV-1-seropositive individuals

Patient*	rIL-2 treatment†	Induration,‡ mm		
		Tuberculin PPD	Candida§	Trichophyton
1	+	0 (0)	11 (11)	6 (3)
2	+	0 (0)	12 (4)	0 (0)
3	+	0 (0)	0 (0)	0 (0)
4	+	0 (0)	10 (0)	0 (0)
5	+	0 (0)	13 (0)	0 (0)
6	+	0 (0)	6 (4)	0 (0)
7	+	0 (0)	11 (3)	0 (0)
8	+	0 (0)	9 (8)	0 (0)
9	+	15 (15)	9 (4)	0 (0)
10	+	0 (0)	10 (5)	0 (0)
11	—	0 (0)	4 (3)	0 (3)
12	—	0 (0)	6 (10)	4 (6)
13¶	—	0 (0)	10 (5)	0 (0)
14	—	0 (0)	5 (3)	6 (5)
15¶	—	0 (0)	11 (8)	0 (0)
16¶	—	0 (0)	3 (0)	0 (0)
17	—	0 (0)	0 (0)	0 (0)
18¶	—	0 (0)	7 (5)	0 (0)
19	—	0 (0)	7 (8)	8 (9)
20	—	0 (0)	0 (0)	0 (0)

\*Patient numbers correspond to those in Table 1.

†Injections of 1, 2, and 5 µg.

‡Induration was determined 48 hr after antigen injection. The previous antigen skin test was carried out &gt;1 year before the present test.

§In a paired comparison test, the enhancement of responsiveness in patients 1–10 was significant; with  $P < 0.005$ . The changes observed in patients 11–20 were not significant.

¶Azidothymidine treatment with or without acyclovir therapy was initiated between the two test periods.

fashion similar to that observed in the immunodeficiency of lepromatous leprosy (6). Our conclusion is supported by the study of Murray *et al.* (12), which provides evidence that the administration of parenteral IL-2 gives rise to large amounts of IFN- $\gamma$  in AIDS patients.

The intensity of the cutaneous response to a skin-test antigen is not as great in HIV-1-seropositive patients as that to recombinant lymphokine. HIV-1-infected patients with prior sensitivity to common antigens may lose or demonstrate fractional reactivity to antigen causing delayed-type hypersensitivity when injected intradermally. In our study this was expressed both as reduced zones of induration and the inability to express MHC class II determinants on the surface of overlying keratinocytes. The expression of IP-10 but not MHC class II antigens on the surface of the keratinocytes suggests that less IFN- $\gamma$  is produced locally in the antigen-responsive site of HIV-infected patients. Since IP-10 can be induced by tumor necrosis factor (TNF) as well as IFN- $\gamma$  (J. V. Ravetch, personal communication), it is possible that TNF is also induced during the local response to antigens in HIV-infected patients. Thus, whereas rIL-2 can stimulate a polyclonal T-cell response with the production of IFN- $\gamma$  and TNF (13, 14), antigen reactivity recruits only a small percentage of CD4<sup>+</sup> T cells, the numbers and activity of which are even further compromised by the disease process.

The ability of rIL-2 to generate an enhanced delayed-type cell-mediated response in the poorly reactive HIV-1-seropositive patients deserves further comment. First, it appears that enhancement is dependent upon prior sensitization of patients to the skin-test antigen and is in keeping with our previous studies in leprosy (11). Second, it is unlikely that sufficient quantities of rIL-2, given at disparate sites, can reach the skin site receiving antigen by a direct vascular or lymphatic route. We suspect that this requires modification of cells passing through the rIL-2 site, their passage back into the circulation and their enhanced homing and reactivity upon entering skin containing an ongoing antigenic challenge. Further studies are required to establish this scenario and to examine the potential of multiple rIL-2 injections and optimal dose range on host cell-mediated reactions. We would hope that this might influence the host's response to secondary intracellular invaders and facilitate defense against opportunistic infections (15). We caution investigators of the potential hazard of inducing HIV-1 replication in cycling T cells with rIL-2 and suggest the combined usage of azidothymidine (AZT) under these conditions.

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# Patients with Multidrug-Resistant Tuberculosis with Low CD4<sup>+</sup> T Cell Counts Have Impaired Th1 Responses<sup>1</sup>

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Multidrug-resistant tuberculosis (MDRTB) has emerged as a challenging clinical problem in both HIV-infected and -uninfected individuals. In this study, immune responses from HIV-negative patients with MDRTB were compared with those of healthy purified protein derivative (PPD)-positive and PPD-negative individuals. These responses were characterized by measuring the proliferation and cytokine production from PBMCs stimulated in vitro with *Mycobacterium tuberculosis*, PPD, or mitogens. MDRTB patients with CD4 counts >500/ $\mu$ l stimulated in vitro with *M. tuberculosis* had similar immune responses (proliferation, IFN- $\gamma$ , and IL-2 production) as the PPD-positive and -negative controls. By contrast, MDRTB patients with CD4 counts <500/ $\mu$ l had markedly deficient immune responses to similar stimuli. In these patients, IFN- $\gamma$  production could be restored by adding IL-12 to the in vitro cultures. IL-12 also caused a striking increase in the amount of IFN- $\gamma$  produced from PBMCs of both PPD-positive and -negative controls. The role of endogenous IL-12 production was also studied. Addition of anti-IL-12 to cultures resulted in a two- to eightfold decrease in IFN- $\gamma$  production in response to PHA stimulation. Inhibition of IFN- $\gamma$  was also observed when cells were stimulated by *M. tuberculosis* and PPD. Using *Staphylococcus aureus* Cowan strain as a mitogenic stimulus, IL-12 p70 was produced in similar amounts in all groups tested. TNF- $\alpha$  production was also assessed from cells stimulated by *M. tuberculosis*. Addition of IL-12 to the cultures did not cause a significant enhancement of TNF- $\alpha$  production. Last, production of IL-10 and IL-4 in response to *M. tuberculosis* and PHA, respectively, was not significantly different among all groups tested. These results suggest that patients with MDRTB tuberculosis with CD4 T cell counts <500/ $\mu$ l have impaired IFN- $\gamma$  and IL-2 responses and might benefit by adjunctive IL-12 therapy. *The Journal of Immunology*, 1997, 158: 492–500.

Recent estimates from the World Health Organization indicate that tuberculosis is the leading cause of mortality from infection in adults (1, 2). Currently, tuberculosis results in approximately 3 million deaths/yr (1, 2). One-third of the world's population is already infected with *Mycobacterium tuberculosis*, and an estimated 90 million new cases of tuberculosis are expected to occur worldwide during the decade from 1990 to 1999 (1, 2). One of the interesting facets of tuberculosis is that exposure to *M. tuberculosis* results in diverse clinical outcomes (3). In the majority of cases, persons exposed to *M. tuberculosis* develop a protective immune response characterized by skin test reactivity to purified protein derivative (PPD);<sup>3</sup> however, for HIV-negative individuals, approximately 10% of those exposed will go on to develop pulmonary disease (1). An even more limited number of patients will go on to develop disseminated or extrapulmonary manifestations of the disease. Recently, a difficult clinical problem has emerged as outbreaks of multidrug-resistant tuberculosis (MDRTB) in both HIV-positive and -negative individuals have been reported worldwide (2).

The increased incidence of MDRTB represents significant problems in terms of both treatment and public health.

There are several mechanisms that might exist independently or together that would lead to the development of MDRTB in HIV-negative individuals: 1) incomplete and/or inappropriate treatment with available chemotherapy; 2) underlying genetic predisposition to disease, such as has been described for disseminated atypical mycobacterial infection; 3) deficiency in host defense due to factors such as poor nutritional status or alcoholism; 4) difference in the virulence among different strains of *M. tuberculosis*; and 5) qualitative or quantitative immune deficiency. There is substantial evidence to support a role for cellular immunity in mediating a protective immune response against tuberculosis (3). Using adoptive transfer studies, it was initially shown that T cells could protect naive animals in response to lethal inocula of *M. tuberculosis* (4). In subsequent studies, IFN- $\gamma$  produced by CD4<sup>+</sup> T cells was shown to play a critical role in protective immunity (5). More definitive evidence for the importance of IFN- $\gamma$  was shown recently in two separate studies in which animals deficient for the IFN- $\gamma$  gene had a rapid and fatal course after infection (6, 7). Finally, IL-12 treatment of mice at the time of infection resulted in increased resistance to infection (8, 9). This effect was abrogated in the absence of IFN- $\gamma$  in vivo, demonstrating that the effects of IL-12 in vivo are probably mediated indirectly through IFN- $\gamma$ .

While IFN- $\gamma$  has been shown to have several different effects on macrophage activation (10), its specific role in mediating killing of intracellular tuberculosis is less clear, especially in studies using human macrophages (11–14). Nevertheless, IFN- $\gamma$  has been shown to be an important immune regulator in studies of patients infected with tuberculosis. In two studies examining the cytokine pattern from pleural fluid, it was shown that IL-2, IFN- $\gamma$ , and TNF- $\alpha$  were all increased preferentially compared with PBMCs (15, 16). Since patients with tuberculous pleuritis have an effective

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<sup>3</sup> Abbreviations used in this paper: PPD, purified protein derivative; MDRTB, multidrug-resistant tuberculosis; SAC, *Staphylococcus aureus* Cowan strain; LAM, liparabinomannan.



immune response, it was inferred that the production of these cytokines may have a role in a protective response. There are several reports comparing the immune responses from PPD<sup>+</sup> control patients who develop a protective immune response with those of patients with pulmonary tuberculosis. In these studies, PBMCs from patients with tuberculosis had reduced production of IL-2 and IFN- $\gamma$  compared with those from healthy, PPD<sup>+</sup> controls (17–20). Since IFN- $\gamma$  was decreased in the patients, studies were performed to determine whether IL-12 was similarly decreased. Interestingly, it was stated that IL-12 protein production was not diminished in PBMCs from tuberculosis patients compared with those from the PPD<sup>+</sup> controls (20); however, in patients who developed an effective immune response, IL-12 was shown to be increased in pleural fluid of patients with tuberculous pleuritis (21). This was compatible with other data showing increased production of IFN- $\gamma$  at the site of disease, consistent with an effective immune response (15, 16).

Studies of IL-12 and IFN- $\gamma$  regulation were extended to analyze HIV-infected individuals with tuberculosis. In this study, Zhang et al. found that PBMCs from HIV-infected patients had deficient proliferative and IFN- $\gamma$  responses compared with those from HIV-negative patients with tuberculosis in response to stimulation with *M. tuberculosis* (22). These responses could be enhanced by addition of IL-12 to the in vitro cultures. In this study, we were interested in characterizing the immune responses from patients with MDRTB at different stages of diseases. We focused our efforts on studying HIV-negative patients with MDRTB, since HIV infection would probably have additional immunologic effects on the response. We found that MDRTB patients with  $>500$  CD4<sup>+</sup> T cells/ $\mu$ l had similar immune responses as those of healthy PPD<sup>+</sup> controls. By contrast, MDRTB patients with  $<500$  CD4<sup>+</sup> T cells/ $\mu$ l had markedly deficient in vitro production of IL-2 and IFN- $\gamma$ . Addition of IL-12 to the in vitro cultures was able to partially restore IFN- $\gamma$  production. These results support a role for exogenous IL-12 in augmenting the cellular immune response in MDRTB patients with low CD4<sup>+</sup> T cell counts.

## Materials and Methods

### Reagents

Complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated human AB sera (Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (2 mM) was used for all stimulations. PHA was purchased from Life Technologies (Grand Island, NY). SAC (*Staphylococcus aureus* Cowan strain, heat-killed) was purchased from Calbiochem (La Jolla, CA). PPD was obtained from Connaught (Swiftwater, PA), and H37Ra (live *Mycobacterium tuberculosis*) was a gift from Drs. Sheldon Morris and Frank Collins of the Food and Drug Administration (Bethesda, MD). Lymphocyte separation medium was purchased from Organon Teknica Corp. (Durham, NC).

### Subjects

Whole blood was obtained by venipuncture from HIV-negative patients with culture proven multidrug-resistant tuberculosis from the National Institutes of Health (Bethesda, MD), The National Jewish Center (Denver, CO), and the Montgomery County Tuberculosis Clinic (Silver Spring, MD). These patients were at various stages in their clinical course, duration of treatment, and extent of disease. PPD<sup>+</sup> and normal controls were also obtained from these facilities. PPD<sup>+</sup> controls were documented to be reactive to tuberculin skin within the last 2 yr. PPD<sup>−</sup> controls were similarly obtained. All specimens were processed and plated into the appropriate cultures immediately or within 24 h of blood collection.

### Recombinant cytokines

Human rIL-12 with a sp. act. of  $5 \times 10^6$  U/mg was purchased from R&D Systems (Minneapolis, MN).

### Antibodies

Goat anti-human IL-12 Ab was purchased from R&D Systems. IL-4R antagonist was provided by Immunex (Seattle, WA). Humanized Abs against the  $\alpha$ -chain (anti-TAC) of the IL-2R were gifts from Dr. John Hakimi (Roche Laboratories, Nutley, NJ).

### Preparation of cells

PBMCs were isolated from heparinized venous blood or buffy coat fractions by lymphocyte separation medium using the density gradient centrifugation method. FACS analysis of the PBMC fraction was then performed, and a CD4 count was calculated based on a concomitant complete blood count. All experiments were performed using one or two MDRTB patients, a PPD<sup>+</sup> control, and a PPD<sup>−</sup> normal control. In each experiment, separate PPD<sup>−</sup> and PPD<sup>+</sup> controls were used.

### Measurement of proliferation in response to stimulation

PBMCs of MDRTB patients and controls were suspended at a concentration of  $1 \times 10^6$  cells/ml in complete medium. A total of  $1 \times 10^5$  cells/(200  $\mu$ l) were added to round-bottom, 96-well plates (Nunc, Roskilde, Denmark) and stimulated in vitro for 5 to 6 days with PPD (final concentration, 0.5  $\mu$ g/ml) and live *M. tuberculosis* (H37Ra; final concentration,  $2 \times 10^4$  organisms/200  $\mu$ l well). PHA-stimulated cultures (final concentration, 1/100 or  $\sim 3$   $\mu$ g/ml) were stimulated in vitro for 3 days. Cultures were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine and harvested after 18 h. Results represent the mean of triplicate wells. The SEM was  $<10\%$  in all experiments.

### Induction of cytokine production by PBMCs

PBMCs ( $2 \times 10^5$  cells/200  $\mu$ l) were added to round-bottom, 96-well plates and stimulated with PHA, PPD, and *M. tuberculosis* (H37Ra) in the presence of various cytokines and/or anticytokines. Supernatants were collected after 5 days of culture (IL-2, IFN- $\gamma$ , and IL-4) and stored at  $-70^\circ\text{C}$  until used. Supernatants were collected at 48 h to measure the production of IL-10, TNF- $\alpha$ , and IL-12. The aforementioned time points were chosen based on kinetic experiments determining the optimal time for specific cytokine production in response to the various stimuli.

### Measurement of cytokine production

IL-2 content was assessed using an IL-2-specific ELISA kit with a lower limit of detection of 16 pg/ml (Genzyme, Cambridge, MA). IL-4 was assessed by an IL-4-specific ELISA with a lower limit of detection of 3 pg/ml (Endogen, Cambridge, MA). IL-10 was determined using a specific ELISA with a lower limit of detection of 16 pg/ml (Endogen). TNF- $\alpha$  was assessed using a specific ELISA with a lower limit of detection of 15 pg/ml (Genzyme). An IL-12-specific ELISA that detects p70 heterodimer was used (R&D Systems). The lower limit of detection for this ELISA was 7.8 pg/ml. IFN- $\gamma$  content was determined by a two-step ELISA assay (23); the lower limit of detection for this assay was 185 pg/ml. Results for all cytokines represent the mean of triplicate wells. The SEM was  $<10\%$  in all experiments.

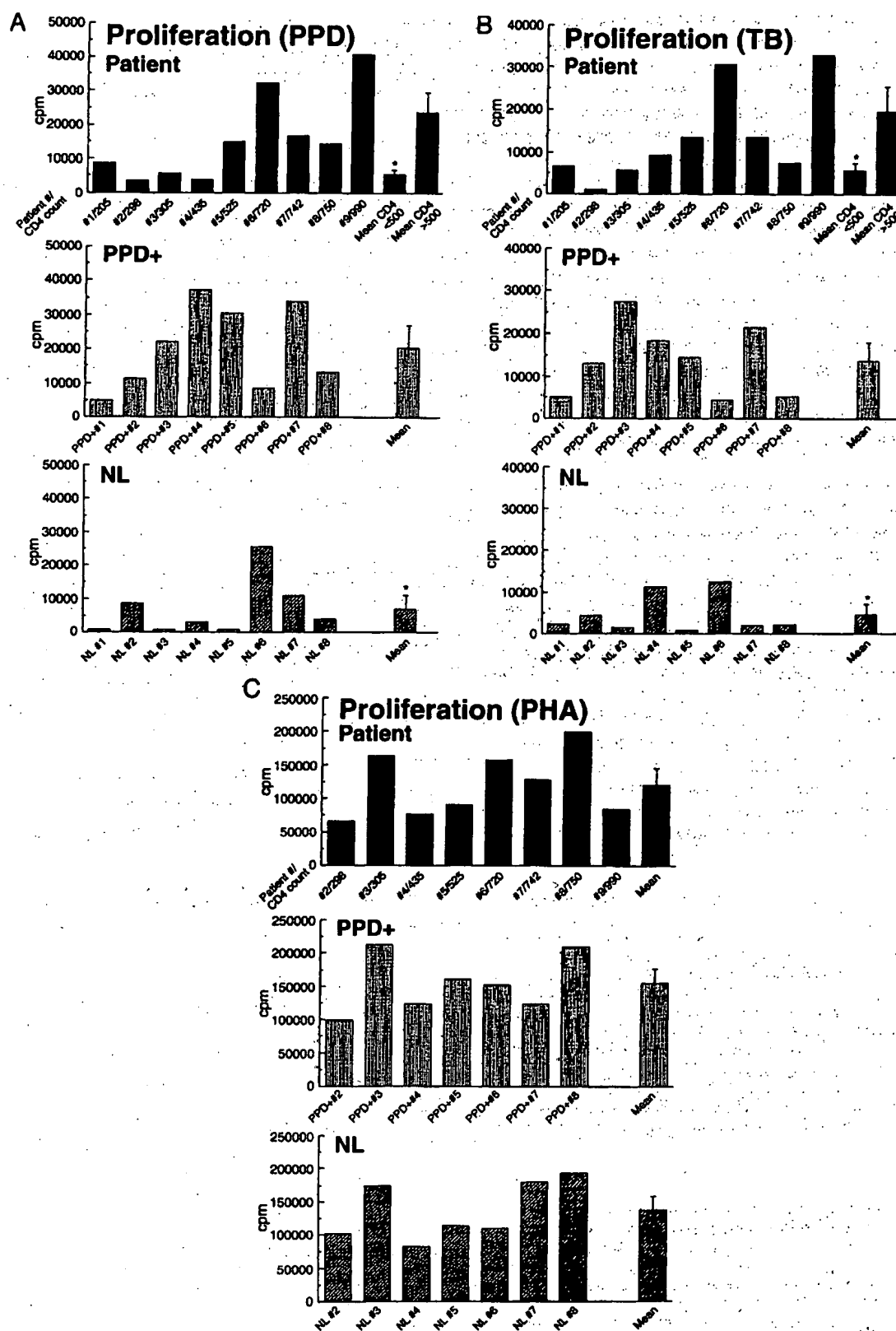
### Statistical analysis

Data in the table and figures are expressed as the mean  $\pm$  SD. Statistical analysis was performed using Student's *t* test, and the results were considered significantly different at  $p < 0.05$ . Data were also evaluated by ANOVA analysis using Statview (Abacus Concepts, Berkeley, CA).

## Results

### Proliferative responses from PBMCs of MDRTB, PPD<sup>+</sup>, and PPD<sup>−</sup> individuals

Proliferation was measured of PBMCs isolated from MDRTB, PPD<sup>+</sup>, and PPD<sup>−</sup> individuals stimulated in vitro with PPD, *M. tuberculosis* (H37Ra), or PHA. MDRTB patients with CD4<sup>+</sup> T cell counts  $>500/\mu$ l all had proliferative responses of  $>10,000$  cpm to either PPD or *M. tuberculosis*, while all patients with CD4<sup>+</sup> T cell counts  $<500/\mu$ l had proliferative responses  $<10,000$  cpm to PPD (Fig. 1A). In healthy control patients with skin test reactivity to PPD (PPD<sup>+</sup>), six of eight individuals had proliferative responses  $>10,000$  cpm. The mean proliferative responses ( $23,841 \pm 11,598$ ) of MDRTB patients with  $>500$  CD4 cells/ $\mu$ l and PPD<sup>+</sup> controls ( $20,227 \pm 12,402$ ) were similar. In contrast,



**FIGURE 1.** Proliferation of PBMCs from MDR TB patients and healthy PPD<sup>+</sup> and PPD<sup>-</sup> individuals stimulated with PPD (A), live *M. tuberculosis* (H37Ra; B), or PHA (C). Fresh PBMCs were added ( $2 \times 10^5/200 \mu\text{l}$ ) to 96-well plates (round-bottom) in triplicate and stimulated for 5 days with PPD ( $0.5 \mu\text{g/ml}$ ) or live avirulent tuberculosis ( $2 \times 10^4$  organisms/ml) or for 3 days with PHA (1/100 final concentration). Following stimulation, cultures were pulsed with  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine and harvested 18 h later. Results are reported as counts per minute. The SEM for the proliferative responses was  $<10\%$  in all experiments. The data are pooled from more than eight experiments. In each experiment, separate PPD<sup>+</sup> and PPD<sup>-</sup> donors were used. Patients 2 and 9 were included in the same experiment. Starred values indicate statistical significance by the two-tailed Student *t* test and ANOVA.

Table 1. MDRTB patients with CD4<sup>+</sup> T cell counts <500/ $\mu$ l have deficient in vitro production of IFN- $\gamma$  and IL-2 in response to PPD and *M. tuberculosis*<sup>a</sup>

PT#/CD4 Count	Immune Parameters				Clinical Parameters		
	IFN- $\gamma$ /TB	IFN- $\gamma$ /PPD	IL-2/TB	IL-2/PPD	Sputum cx	Hypoxia	Malnourished
Good responders							
#5/525	3084	6000	276	671	-	-	-
#6/720	2463	3284	1800	378	-	-	-
#7/742	3219	1899	525	1140	+	-	-
8/750	9396	6375	840	660	-	-	-
9/990	4436	3150	170	183	-	-	-
Mean	4520 $\pm$ 2520	4142 $\pm$ 1949	722 $\pm$ 586	606 $\pm$ 362			
Poor responders							
#1/205	0	0	74	61	-	+	+
#2/298	0	0	0	0	-	+	+
#3/305	495	0	355	341	+	-	-
#4/435	295	0	147	39	+	-	+
Mean	198 $\pm$ 242	0	144 $\pm$ 153	110 $\pm$ 156			

<sup>a</sup> Fresh PBMCs from MDRTB patients and healthy PPD<sup>+</sup> and PPD<sup>-</sup> individuals were added ( $2 \times 10^5/200 \mu$ l) in triplicate to 96-well plates (round bottom) and stimulated with *M. tuberculosis* ( $2 \times 10^4$  organisms/ml) or PPD (0.5  $\mu$ g/ml). Supernatants were collected after 5 days, and IFN- $\gamma$  content was measured by ELISA. For IL-2 production, Ab against IL-2R $\alpha$  (anti-TAC) (10  $\mu$ g/ml) was added to cultures to prevent consumption of IL-2. Values shown are in pg/ml. The data represent eight separate experiments. The SEM for IL-2 and IFN- $\gamma$  was <10% in all experiments. Two-tailed Student's *t* test and ANOVA were used for statistical analysis.

only two individuals who reported no known exposure to tuberculosis and were negative for PPD skin test reactivity by history showed a proliferative response of >10,000 cpm. The majority of these subjects had proliferative responses of approximately 5000 cpm or less, and their mean proliferative response to PPD (6756  $\pm$  8523) was similar to that of the MDRTB patients with <500 CD4 cells/ $\mu$ l (5467  $\pm$  2404). Similar results were observed using live *M. tuberculosis* as an Ag (Fig. 1B). Moreover, there was a statistically significant decrease in proliferation in response to either PPD or live tuberculosis ( $p < 0.018$  and  $p < 0.028$ , respectively; Fig. 1, A and B) in patients with <500 CD4 cells/ $\mu$ l compared with those patients with CD4 counts of >500 cells/ $\mu$ l and PPD<sup>+</sup> controls. To assess the proliferative response under maximal conditions, PBMCs were stimulated with the polyclonal mitogen PHA (Fig. 1C). Similar proliferative responses were seen in all groups tested, including MDRTB patients with lower CD4<sup>+</sup> T cell counts. Thus, MDRTB patients with CD4 counts of >500/ $\mu$ l had Ag-specific proliferative responses similar to those of healthy PPD<sup>+</sup> controls, while patients with CD4 counts <500/ $\mu$ l were relatively deficient.

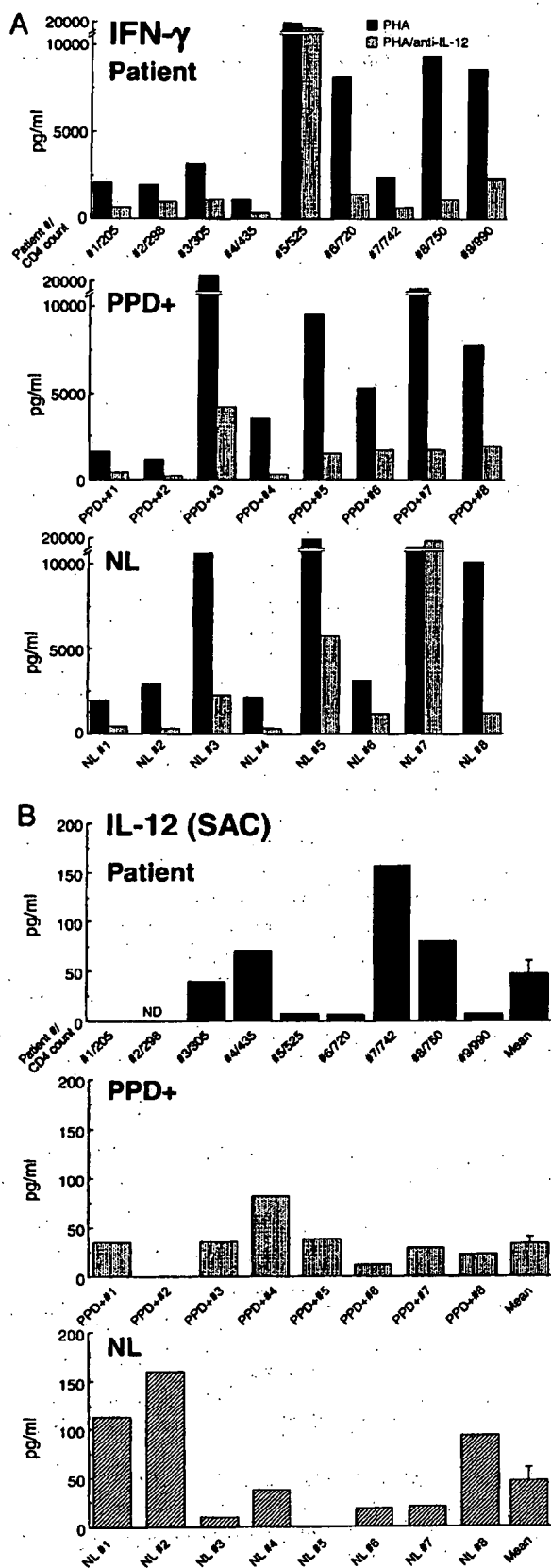
#### MDRTB patients with CD4<sup>+</sup> T cell counts <500/ $\mu$ l have decreased production of IL-2 and IFN- $\gamma$ in response to specific tuberculin Ag

Several studies in both murine and human experimental models of tuberculosis have shown IFN- $\gamma$  to be critical in mediating a protective immune response (3). We measured IFN- $\gamma$  production from PBMCs of MDRTB patients following in vitro stimulation with *M. tuberculosis* or PPD (Table I). IFN- $\gamma$  production from patients with CD4 counts <500/ $\mu$ l was statistically lower (mean, 198  $\pm$  242 pg/ml) than that of patients with CD4 counts >500 (mean, 4520  $\pm$  2520) in response to *M. tuberculosis* ( $p = 0.009$ ). Similar quantitative and statistical data were seen using PPD as an Ag. In contrast, significant amounts of IFN- $\gamma$  were produced from patients with CD4 counts <500/ $\mu$ l in response to PHA (Fig. 2A), demonstrating that under optimal stimulatory conditions, there is a quantitative, but not an absolute qualitative, defect in IFN- $\gamma$  production in these patients. There are several factors that might affect the in vitro production of IFN- $\gamma$ . We and others have previously shown that the presence of IL-2 is important for optimal in vitro production of IFN- $\gamma$  (23). To determine whether IL-2 was also diminished in MDRTB patients with low CD4<sup>+</sup> T cell counts, we mea-

sured IL-2 by stimulating cells in vitro in the presence of anti-IL-2R  $\alpha$ -chain Ab (anti-TAC) to prevent consumption of IL-2. Patients with CD4 counts >500/ $\mu$ l had significantly higher production of IL-2 in response to either *M. tuberculosis* (722  $\pm$  586 pg/ml) or PPD (606  $\pm$  362 pg/ml) compared with patients with CD4 counts <500/ $\mu$ l (144  $\pm$  153 and 110  $\pm$  156 pg/ml in response to *M. tuberculosis* and PPD, respectively). Table I also lists three clinical parameters that were followed in these patients. Patients with CD4 counts <500 had a more severe clinical course, based on having at least two of these parameters. Furthermore, patients were arbitrarily grouped into "good" and "poor" responders based on clinical course and relative production of IFN- $\gamma$  and IL-2. In addition to the clinical parameters listed in Table I, three of four MDRTB patients with CD4<sup>+</sup> T cell counts <500 had a history of infection extending over 3 yr, with multiple relapses. The two patients with the lowest CD4<sup>+</sup> T cell counts had the most extensive pulmonary disease, with significant lung destruction leading to hypoxia. Thus, in this small number of patients, clinical severity seemed to correlate with lower CD4 counts and diminished in vitro cellular immune responses.

#### Characterization of endogenous IL-12 and its role in IFN- $\gamma$ production in vitro

As stated above, one of the major regulators of IFN- $\gamma$  production is IL-12. We were interested in determining whether MDRTB patients with low CD4 cell counts had a decrease in endogenous IL-12 production that would explain their deficient IFN- $\gamma$  responses. Endogenous IL-12 was first assessed indirectly by measuring IFN- $\gamma$  production in the presence of neutralizing Ab to IL-12. We used PHA as a stimulus to induce in vitro production of IFN- $\gamma$ , since MDRTB patients with low CD4 counts produced little or no IFN- $\gamma$  in response to *M. tuberculosis*. Furthermore, the magnitude of the IFN- $\gamma$  response to PHA would provide a greater window for examining the role of endogenous IL-12. As shown in Figure 2A, the presence of anti-IL-12 in cultures resulted in a two- to eightfold decrease in the amount of IFN- $\gamma$  produced, suggesting that endogenous IL-12 is responsible for a majority of the IFN- $\gamma$  produced in all patients in response to a mitogenic stimulus. Similarly, in those patients and controls who had detectable IFN- $\gamma$  (185 pg/ml) in response to *M. tuberculosis*, addition of anti-IL-12 caused a 50 to 100% decrease in IFN- $\gamma$  production (data not shown). We then measured IL-12 p70 heterodimer directly from



**FIGURE 2.** Characterization of endogenous IL-12 production and its effect on IFN- $\gamma$  production. **A**, Fresh PBMCs from MDRTB patients and healthy PPD $^{+}$  and PPD $^{-}$  individuals were added ( $2 \times 10^5/200 \mu\text{l}$ ) in triplicate to 96-well plates and stimulated with PHA (1/100, final concentration) in the presence or absence of anti-IL-12 (3  $\mu\text{g/ml}$ ). Super-

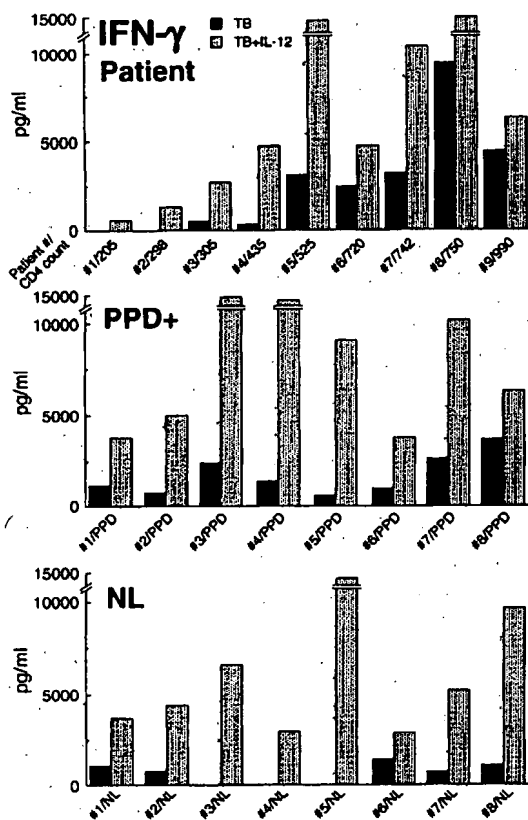
natants were collected after 5 days, and IFN- $\gamma$  content was measured by ELISA. Values shown are reported as picograms per milliliter. The data represent eight separate experiments. The SEM was  $<10\%$  in all experiments. **B**, Fresh PBMCs from MDRTB patients and healthy PPD $^{+}$  and PPD $^{-}$  individuals were added ( $2 \times 10^5/200 \mu\text{l}$ ) in triplicate to 96-well plates (round-bottom) and stimulated with SAC (0.0075%/volume). Supernatants were collected after 48 h, and IL-12 content was measured by ELISA. The sensitivity of the ELISA is 7.8 pg/ml. Values shown are reported as picograms per milliliter. The data represent eight separate experiments. The SEM was  $<10\%$  in all experiments.

#### Exogenous IL-12 significantly increases IFN- $\gamma$ production in response to tuberculosis-specific Ag

Since patients with CD4 counts  $<500/\mu\text{l}$  have deficient production of IFN- $\gamma$ , we were interested in whether addition of IL-12 to cultures could increase IFN- $\gamma$  production. To assess the effect of IL-12 on Ag-specific IFN- $\gamma$  production, we subtracted the amount of IFN- $\gamma$  produced in cultures with IL-12 alone from that produced in cultures with IL-12 plus Ag. In the experiments shown in Figure 3, the presence of exogenous IL-12 resulted in a significant increase in IFN- $\gamma$  production in all groups tested. Moreover, the presence of IL-12 was able to induce IFN- $\gamma$  in MDRTB patients with CD4 counts  $<500$  who had minimal to undetectable baseline production of IFN- $\gamma$ . Similar data were obtained using PPD as an Ag. There are several reasons why IL-12 was able to cause an increase in Ag-specific IFN- $\gamma$  from normal patients not previously exposed to *M. tuberculosis*. We have outlined these potential mechanisms in Discussion.

#### Addition of IL-12 to *in vitro* cultures stimulated with *M. tuberculosis* does not increase the production of TNF- $\alpha$

Studies from both murine and human experimental models have shown TNF- $\alpha$  to be an important immune mediator in the host response to tuberculosis infection (24–26). TNF- $\alpha$  production was measured from PBMCs stimulated *in vitro* with *M. tuberculosis* (Fig. 4). In contrast to data showing IFN- $\gamma$  and IL-2 to be impaired in the MDRTB patients with low CD4 $^{+}$  T cell counts, TNF- $\alpha$  was produced in similar amounts by all patients with MDRTB regardless of their CD4 $^{+}$  T cell counts. Moreover, the amount of TNF- $\alpha$  produced by the patients was not appreciably different from that produced by the PPD $^{+}$  and PPD $^{-}$  controls. The TNF- $\alpha$  produced by the PPD $^{-}$  subjects is consistent with data showing that lipopolysaccharide (LPS), a major component of mycobacterial cell walls, is a potent inducer of TNF- $\alpha$  from macrophages and PBMCs of both patients with tuberculosis and normal individuals (27, 28). Since macrophages are a major producer of TNF- $\alpha$  in



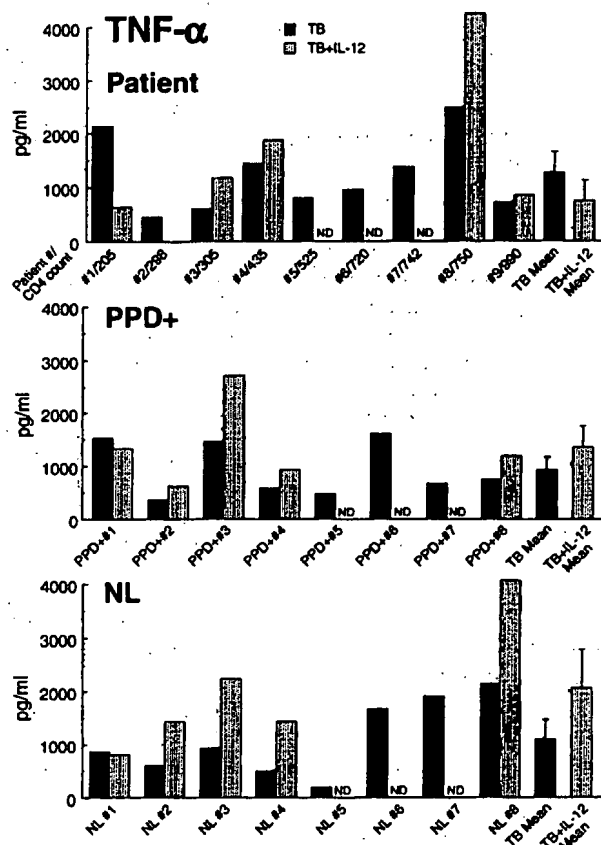
**FIGURE 3.** Addition of IL-12 increases IFN- $\gamma$  production in vitro from PBMCs stimulated with live *M. tuberculosis* (H37Ra). Fresh PBMCs from MDRTB patients and healthy PPD<sup>+</sup> and PPD<sup>-</sup> individuals were added ( $2 \times 10^5/200 \mu\text{l}$ ) in triplicate to 96-well plates and stimulated with *M. tuberculosis* ( $2 \times 10^4$  organisms/ml) in the presence or the absence of IL-12 (1 ng/ml). Supernatants were collected after 5 days, and IFN- $\gamma$  content was measured by ELISA. The sensitivity of the assay was between 100 and 200 pg/ml. Values shown are reported as picograms per milliliter. The data represent eight separate experiments. The SEM was <10% in all experiments.

response to LAM, and all the MDRTB patients had similar numbers of macrophages, it is not surprising that TNF- $\alpha$  was produced in similar amounts. Finally, addition of polymyxin B to the cultures stimulated with *M. tuberculosis* did not significantly reduce the TNF- $\alpha$  response, providing evidence against LPS contamination in the Ag preparation (data not shown).

Since IL-12 is a potent inducer of IFN- $\gamma$ , it has significant potential as an adjunctive therapeutic agent to existing drug therapy in patients who have diminished IFN- $\gamma$  responses and difficult clinical courses; however, previous studies by Orange et al. have shown that mice infected with LCMV and treated with IL-12 had dramatic toxicity mediated by increased TNF- $\alpha$  production (29). To this end, we determined the effect of exogenous IL-12 on in vitro production of TNF- $\alpha$ . Surprisingly, TNF- $\alpha$  production was not appreciably increased when IL-12 was added to the in vitro cultures in any of the groups tested.

#### Production of IL-4 and IL-10 is similar in MDRTB patients and controls

It has been suggested that decreased Th1 responses in patients with tuberculosis is due to the production of cytokines capable of down-regulating IFN- $\gamma$  production (22). These down-regulatory cytokines include IL-4, IL-10, and TGF- $\beta$  (30). When we assessed the



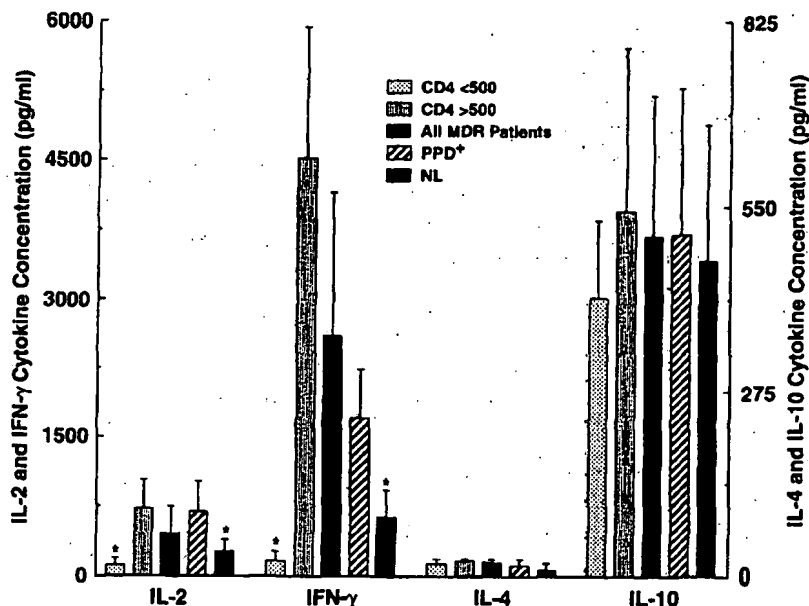
**FIGURE 4.** Addition of exogenous IL-12 has little effect on TNF- $\alpha$  production from PBMCs of MDRTB patients and PPD<sup>+</sup> and PPD<sup>-</sup> individuals stimulated in vitro with *M. tuberculosis*. Fresh PBMCs from MDRTB patients and healthy PPD<sup>+</sup> and PPD<sup>-</sup> individuals were added ( $2 \times 10^5/200 \mu\text{l}$ ) in triplicate to 96-well plates and stimulated with *M. tuberculosis* ( $2 \times 10^4$  organisms/ml) in the presence or the absence of IL-12 (1 ng/ml). Supernatants were collected after 2 days, and TNF- $\alpha$  content was measured by ELISA. The sensitivity of the assay was 15 pg/ml. The data represent eight separate experiments. In five of these experiments, IL-12 was added to the cultures. The SEM was <10% in all experiments. ND, not done.

production of IL-4 and IL-10 in response to various stimuli, IL-4 could not be detected above the lower limit of the ELISA (<3 pg/ml) from PBMCs stimulated with *M. tuberculosis* (Fig. 5). Using PHA as a stimulus, IL-4 was induced in low amounts from all groups tested. In addition, IL-10, a cytokine produced by T cells and macrophages that inhibits IFN- $\gamma$  production, was detected in similar amounts from supernatants of PBMCs stimulated with *M. tuberculosis* from all groups (Fig. 5). Figure 5 also shows the mean production of IL-4 and IL-10 compared with that of IL-2 and IFN- $\gamma$  in all groups tested. This summary figure clearly demonstrates that MDRTB patients with CD4<sup>+</sup> T cells counts <500/ $\mu\text{l}$  have impaired production of IL-2 and IFN- $\gamma$ , as discussed above.

#### Discussion

Deficiencies in both the qualitative and quantitative aspects of the immune response have been shown to have an impact on clinical outcome in patients infected with tuberculosis. The cellular immune response involves activation of T cells with production of cytokines such as IL-2 and IFN- $\gamma$ . In several studies, proliferation and IL-2 production from PBMCs have been found to be decreased in patients with active tuberculosis and advanced disease (31, 32).

**FIGURE 5.** The productions of IL-4 and IL-10 are similar in MDRTB patients and controls, in contrast to those of IL-2 and IFN- $\gamma$ . Fresh PBMCs ( $2 \times 10^5/200 \mu\text{l}$ ) from MDRTB patients and healthy PPD $^+$  and PPD $^-$  individuals were added in triplicate to 96-well plates and stimulated with *M. tuberculosis* ( $2 \times 10^4$  organisms/ml). For assessment of IL-10 production, supernatants were collected 2 days after stimulation. To assess IL-2 and IFN- $\gamma$  content, supernatants were collected 5 days after stimulation. For IL-4 production,  $2 \times 10^5/200 \mu\text{l}$  PBMCs from all groups were stimulated with PHA (1/100 final concentration) in the presence of Ab to the human IL-4R (10  $\mu\text{g/ml}$ ). Supernatants were harvested 5 days after stimulation and assayed for IL-4 content. The lower limit of detection for IL-4 was 3 pg/ml. Values shown are reported as picograms per milliliter. The data represent eight separate experiments. The SEM was  $<10\%$  in all experiments. Starred values indicate statistical significance.



More recently, Zhang et al. showed that purified CD4 $^+$  cells from TB patients had decreased expression of IL-2 mRNA compared with healthy PPD $^+$  controls, suggesting a qualitative defect in IL-2 production (20). In this report, MDRTB patients with CD4 $^+$  T cell counts  $>500/\mu\text{l}$  had similar in vitro proliferative responses to PPD or *M. tuberculosis* as those of PPD $^+$  controls (Fig. 1). Conversely, MDRTB patients with CD4 $^+$  T cell counts  $<500/\mu\text{l}$  had markedly deficient proliferative responses to similar Ags. Proliferation is often a functional surrogate to IL-2 production. Moreover, IL-2 production from the MDRTB patients was markedly diminished in response to the same Ags compared with that in patients with CD4 counts  $>500/\mu\text{l}$  or that in PPD $^+$  controls. An exception to this was MDRTB patient 9 (CD4 $^+$  count, 990); this patient had the highest proliferative response but relatively low IL-2 production. This may reflect incomplete blockade of IL-2 consumption by the anti-TAC Ab in the cultures. Thus, our results are similar to those reported above, showing a defect in IL-2 production and proliferation. One possibility is that the diminished IL-2 and IFN- $\gamma$  responses are due to the lower number of Ag-specific CD4 $^+$  T cells in those patients with CD4 $^+$  T cell counts  $<500/\mu\text{l}$ ; however, we cannot rule out a qualitative defect as well, which has been suggested by others (31, 32). To resolve the issue of whether this is a qualitative and/or a quantitative defect would require using the same number of purified CD4 $^+$  T cells from patients and controls as responder cells in the stimulations. We were prevented from conducting these experiments by the requirement for a large number of cells from these very ill patients.

In addition to IL-2, IFN- $\gamma$  has been thought to be a critical regulator in mediating a protective response against intracellular pathogens such as tuberculosis. In this study, IFN- $\gamma$  production was significantly lower in patients with CD4 counts of  $<500/\mu\text{l}$  than in patients with CD4 counts  $>500/\mu\text{l}$  and PPD $^+$  controls in response to TB-specific Ag (Table I and Fig. 3). This is similar to the IL-2 and proliferation data we have presented. There are many potential mechanisms to explain why IFN- $\gamma$  is deficient in these patients. 1) Patients with low CD4 $^+$  T cell counts have a decrease in the number of Ag-specific cells capable of producing IFN- $\gamma$ . In earlier studies measuring IFN- $\gamma$  production from patients with tuberculosis, it was shown that IFN- $\gamma$  was deficient in some patients (17, 18). 2) We have previously shown that the presence of IL-2 is

required for optimal IFN- $\gamma$  production in vitro (23). In some of the experiments reported here, anti-TAC was added to cultures stimulated with *M. tuberculosis* or PPD. In these experiments, IFN- $\gamma$  production was markedly reduced (data not shown). 3) The presence of down-regulatory cytokines such as IL-4, IL-10, or TGF- $\beta$  could be involved in inhibiting IFN- $\gamma$  production. Zhang et al. reported no enhancement of type 2 cytokines in HIV-infected patients with tuberculosis (22); however, addition of anti-IL-10 to cultures caused an increase in both IFN- $\gamma$  production and proliferation in HIV-infected tuberculosis patients. Interestingly, these patients produced IL-10 in response to specific Ag in similar amounts as did the HIV-negative tuberculosis patients. In addition, anti-IL-10 had a minimal effect on increasing the proliferative response of these patients, suggesting that IL-10 is playing a more prominent role in the down-regulation of responses in HIV-infected tuberculosis patients (22). Our results show that MDRTB patients produce similar amounts of IL-10 and IL-4 as the controls (Fig. 5). We did not test the effect of adding anti-IL-4 or anti-IL-10 to the cultures to determine whether IFN- $\gamma$  was increased in the MDRTB patients with low CD4 $^+$  T cell counts. Although IL-4 was produced in similar amounts in all groups in response to PHA, we do not know whether there is a bias toward IL-4 production in response to specific Ag due to our failure to detect it in our ELISA. 4) IL-12 is a potent regulator of IFN- $\gamma$  and is thought to be involved in augmenting IFN- $\gamma$  production at the site of disease for tuberculosis (21). Thus, a defect in IL-12 production may account for the lack of IFN- $\gamma$  in the MDRTB patients with low CD4 $^+$  T cell counts (see below).

IL-12 has been shown to be a potent regulator of IFN- $\gamma$  production in many experimental models. In studying patients with tuberculous pleuritis, Zhang et al. noted that IL-12 was increased in pleural fluid from patients compared with the levels in peripheral blood (21). We first assessed the role of endogenous IL-12 to determine whether the deficient IFN- $\gamma$  responses in the MDRTB patients were attributable to a defect in IL-12 production. Similar to other studies, we were unable to detect appreciable amounts of IL-12 from PBMCs of MDRTB patients stimulated with *M. tuberculosis*. Thus, PBMCs were stimulated with SAC, a potent activator of human macrophages, to elicit IL-12 production. In response to SAC, there were no significant differences in the

production of IL-12 p70 heterodimer in the MDRTB patients (regardless of CD4 count) compared with that in the control populations (Fig. 2B). These results could be explained by the fact that SAC might act directly on macrophages to produce IL-12 independent of CD4<sup>+</sup> T cells. Since most of the MDRTB patients had normal numbers of macrophages (data not shown), it is not surprising that IL-12 production in response to a T-independent stimulus would be similar to the PPD<sup>+</sup> and PPD<sup>-</sup> controls. Interestingly, when PBMCs were stimulated with SAC *M. tuberculosis*, there was a marked decrease in the amount of IL-12 p70 produced compared with that by cultures stimulated with SAC alone. Addition of anti-IL-10 to cultures stimulated with SAC and *M. tuberculosis* fully restored the IL-12 production seen with SAC alone (data not shown). These results suggest that the *M. tuberculosis* (H37Ra) used for stimulation preferentially induced IL-10, which impaired IL-12 production. Studies are underway to evaluate various live virulent strains isolated from patients to determine their effects on IL-10 and IL-12 production from PBMCs and purified macrophages. The role of endogenous IL-12 was assessed indirectly by measuring IFN- $\gamma$  production from cultures stimulated with PHA in the presence of a neutralizing Ab to IL-12. In the experiments shown in Figure 2A, the presence of anti-IL-12 caused a two- to eightfold reduction in the amount of IFN- $\gamma$  produced. Interestingly, there appeared to be a greater degree of inhibition in MDRTB patients with CD4<sup>+</sup> T cell counts >500/ $\mu$ l and PPD<sup>+</sup> and PPD<sup>-</sup> controls than in the patients with low CD4 counts. The presence of anti-IL-12 in cultures stimulated with *M. tuberculosis* or PPD also caused a striking inhibition of IFN- $\gamma$  in those groups in which IFN- $\gamma$  was detected (data not shown). These results demonstrate that endogenous IL-12 has an important role in IFN- $\gamma$  production.

One of our primary interests in undertaking these studies was to determine whether MDRTB patients had deficient IFN- $\gamma$  responses and, if so, whether they could be restored by adding exogenous IL-12 to cultures. Addition of IL-12 to cultures resulted in a striking increase in IFN- $\gamma$  production in MDRTB patients and controls (Fig. 3). Moreover, it induced IFN- $\gamma$  production in several patients with MDRTB (CD4 counts <500/ $\mu$ l) who did not produce any IFN- $\gamma$  in response to *M. tuberculosis* or PPD as well as in the PPD<sup>-</sup> controls. There are several mechanisms that could explain the increase in IFN- $\gamma$  production by the PPD<sup>-</sup> controls in response to tuberculosis-specific Ag. In other experimental models, IL-12 is especially potent in augmenting responses under limiting stimulatory conditions (23, 33). It has been shown to act as a costimulatory molecule for both proliferation and IFN- $\gamma$  production (34, 35). It is possible that normal individuals are exposed to environmental mycobacterial Ags that are cross-reactive with the Ags used in this study as stimuli. Additionally, there are different cells, such as CD4<sup>-</sup>CD8<sup>-</sup> (double negative) (36) and CD8<sup>+</sup> $\alpha\beta$  (37) CD1-restricted T cells from normal donors, that have been shown to produce IFN- $\gamma$  in response to nonpeptide Ags such as mycolic acids and lipoglycans. Furthermore, human  $\gamma\delta$  T cells from tuberculin-positive donors were found to produce IFN- $\gamma$  and IL-2 in response to tuberculosis Ags (38, 39). In addition, there is evidence that *M. tuberculosis* acts like a superantigen in augmenting the proliferative response of PBMCs from normal donors (40). Finally, NK cells have been shown to be a potent source of IFN- $\gamma$  in the presence of IL-12. In this regard, it is possible that tuberculosis Ags can elicit IFN- $\gamma$  production from NK cells that is further increased in the presence of IL-12.

TNF- $\alpha$  has been demonstrated to have an important role in immunity against tuberculosis (24–26). A 30-kDa Ag secreted from *M. tuberculosis* has been shown to be a strong inducer of TNF- $\alpha$  in normal human monocytes (41). LAM, a major cell wall com-

ponent of *M. tuberculosis*, was also shown to stimulate human macrophages from PPD<sup>+</sup> donors to produce TNF- $\alpha$  (27, 28). In the studies reported here, TNF- $\alpha$  is produced in similar amounts from all patients with MDRTB regardless of CD4 count. In addition, the PPD<sup>+</sup> and PPD<sup>-</sup> controls produced similar quantities of TNF- $\alpha$  in response to *M. tuberculosis*. The finding that all the MDRTB patients produced similar amounts of TNF- $\alpha$  as the controls is consistent with the data, in that the numbers of macrophages were similar in all groups regardless of CD4 T cell counts (data not shown). Since addition of IL-12 to the cultures caused a striking increase in IFN- $\gamma$ , we wished to evaluate its effect on TNF- $\alpha$  production. Interestingly, addition of exogenous IL-12 to cultures stimulated with *M. tuberculosis* caused a modest increase in TNF- $\alpha$  production in both MDRTB patients and controls. Since we showed that IL-12 caused a more substantial increase in IFN- $\gamma$  from the same cultures, TNF- $\alpha$  may be induced directly by *M. tuberculosis*. Furthermore, addition of anti-IL-12 or anti-IFN- $\gamma$  to cultures did not appreciably diminish TNF- $\alpha$  production (data not shown). Moreover, MDRTB patients with CD4<sup>+</sup> T cell counts <500/ $\mu$ l produced minimal amounts of IFN- $\gamma$ , but had similar production of TNF- $\alpha$  as patients with higher CD4 counts. Our data are supported by the observation that IFN- $\gamma$ -deficient mice infected with *Mycobacterium bovis* had similar mRNA expression of TNF- $\alpha$  as the wild-type controls (7). These results support the idea that *M. tuberculosis* can induce TNF- $\alpha$  directly from PBMCs independent of IL-12 or IFN- $\gamma$ .

Studies in both murine and human experimental models of *M. tuberculosis* indicate that CD4 T cells play a crucial role in protective immunity to infection. Recently, the CD4 count was demonstrated to be the sole independent predictor of survival in HIV-related tuberculosis (42). Additionally, lymphocytopenia in the setting of non-HIV-related tuberculosis has been well described (43, 44). In a selected group of patients, lymphocytopenia resolved following successful anti-tuberculosis treatment (45). In the study of MDRTB patients reported here, CD4 lymphocytopenia appeared to correlate with the severity of disease and low IL-2/IFN- $\gamma$  production. Whether CD4 lymphocytopenia is a cause or an effect of the severity of the disease is unknown. Moreover, the mechanism of CD4 lymphocytopenia is also unknown, although there is recent evidence that patients with idiopathic CD4<sup>+</sup> T lymphocytopenia have accelerated T cell apoptosis (46). We hope to perform follow-up studies in MDRTB patients with low CD4<sup>+</sup> T cell counts pending recovery.

This study confirms previous work showing that IL-12 can enhance IFN- $\gamma$  production in an Ag-specific manner. Since treatment of MDRTB requires prolonged therapy with multiple pharmacologic agents, our results suggest that these patients, especially those who present with low CD4<sup>+</sup> T cell counts, might be candidates for adjunctive cytokine therapy. In this regard, Holland et al. have reported success in treating patients with refractory disseminated nontuberculous mycobacterial infection with IFN- $\gamma$  in combination with conventional chemotherapy (47). All these patients were shown to have defective production of IFN- $\gamma$  in response to PHA, and four of the seven patients had idiopathic CD4 lymphocytopenia. One caveat to using IL-12 as a therapeutic agent is its ability to mediate a pro-inflammatory response. In an experimental model of viral infection, Orange et al. have shown that high dose IL-12 treatment leads to overproduction of TNF- $\alpha$ , with a subsequent increase in mortality (29). While TNF- $\alpha$  has been shown to have a critical role in the protective immune response in tuberculosis, there appears to be a narrow therapeutic window in which too much TNF- $\alpha$  may be harmful (26). It is both interesting and surprising that addition of IL-12 augmented IFN- $\gamma$  production but did not appreciably increase TNF- $\alpha$  production in response to

*M. tuberculosis*. If IL-12 were to act in a similar manner in these patients in vivo, it would offer a promising therapeutic to enhance specific immunity while limiting toxicity.

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# Synergistic Enhancement of Cell-Mediated Immunity by Interleukin-12 Plus Interleukin-2: Basis for Therapy of Cutaneous T Cell Lymphoma

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Cutaneous T cell lymphoma is a clonally derived, skin-invasive malignancy of CD4<sup>+</sup> T lymphocytes with the phenotype of mature helper T cells. Previous work has demonstrated that the Sézary form, or typically leukemic form of cutaneous T cell lymphoma, is characterized by prominent immunologic defects, including depressed cell-mediated immunity associated with marked defects in the production of interleukin-12 and other type 1 helper T cell cytokines. Recent clinical trials with recombinant human interleukin-12 for cutaneous T cell lymphoma have demonstrated that it is a potent therapeutic agent, which induces cytotoxic T cell responses. Nevertheless, a high rate of refractoriness to recombinant human interleukin-12 occurred in these studies that may be related to the downmodulation of interleukin-12 receptor expression by chronic interleukin-12 use. In an effort to enhance the overall response rate and to overcome the refractoriness to recombinant human interleukin-12 therapy, we studied the immunologic effects *in vitro* of adding interleukin-2 to interleukin-12 as a model to achieve these goals. We examined the stimulation of interferon- $\gamma$  production, natural killer cell activity and interleukin-12

receptor expression by T cells of cutaneous T cell lymphoma patients. The addition of interleukin-12 to cutaneous T cell lymphoma patient peripheral blood cells resulted in the production of interferon- $\gamma$  (mean = 7914 pg per ml  $\pm$  2161, n = 15) as did interleukin-2 alone (mean = 7222 pg per ml  $\pm$  2228, n = 15). Importantly, the addition of interleukin-2 to the interleukin-12 synergistically enhanced the levels of interferon- $\gamma$  produced (mean = 16 792 pg per ml  $\pm$  2492 n = 15) (p < 0.01). Similarly, addition of interleukin-2 to interleukin-12 synergistically enhanced both the natural killer cell activity of 15 cutaneous T cell lymphoma patients as well as T cell surface interleukin-12 receptor expression in comparison with the effects of interleukin-12 or interleukin-2 alone. Thus, interleukin-2 plus interleukin-12 unequivocally produces the synergistic enhancement of multiple parameters of cell-mediated immunity as well as upmodulating interleukin-12 receptor expression; this indicates that protocols combining these two potent immune enhancing cytokines may have added therapeutic benefit for cutaneous T cell lymphoma. **Key words:** CTCL T cell/IL-2/IL-12/IL-12 receptors. *J Invest Dermatol* 118:366-371, 2002

Cutaneous T cell lymphoma (CTCL) is a lymphoproliferative disorder typically characterized by invasion of the skin with clonally derived malignant CD4<sup>+</sup> lymphocytes that bear the phenotype of mature helper T cells (Edelson, 1980; Haynes *et al*, 1981; Weiss *et al*, 1985). Sézary syndrome is a more advanced stage of CTCL. It usually presents with diffuse skin involvement, generalized lymphadenopathy, and the presence of circulating malignant CD4<sup>+</sup>/CD45RO<sup>+</sup> T cells (Haynes *et al*, 1981; Rook *et al*, 1997). Sézary syndrome is also characterized by a variety of immunologic

abnormalities, including depressed cell-mediated cytotoxicity, deficient responsiveness of T cells to antigens and mitogens, elevated serum IgE and IgA, as well as eosinophilia (Rook *et al*, 1993; Rook and Heald, 1995). Recent studies of the nature of the malignant T cells have provided evidence that these cells are at least partially responsible for the generation of the immune defects by way of production of immunosuppressive T helper type (Th) 2 cytokines such as interleukin (IL)-4, IL-5, and IL-10, and depressed Th1 type cytokines (Vowels *et al*, 1992, 1994; Rook and Heald, 1995; Rook *et al*, 1995; Dummer *et al*, 1996).

In contrast to increased Th2 cytokine production, we have also observed a marked depression in production of Th1 cytokines, IL-2 and interferon (IFN)- $\gamma$ , by peripheral blood mononuclear cells (PBMC) from Sézary syndrome patients (Vowels *et al*, 1992, 1994; Rook *et al*, 1993). As Th1 cytokines are critical for the development of normal cell-mediated immunity and are, therefore, necessary for the genesis of anti-tumor immunity, this finding may account for the progressive depression in cell-mediated immunity

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Abbreviations: CTL, cytotoxic T lymphocyte; rhIL-12, recombinant human interleukin-12.

associated with the progression of clinical disease. Thus, in the setting of depressed Th1 cytokine production and increased Th2 cytokines, which can suppress cell-mediated immunity (Rook *et al.*, 1993), these observations help us to understand further the basis for depressed cell-mediated immunity in advanced CTCL.

IL-12 is a monocyte/macrophage derived cytokine, which is a powerful inducer of IFN- $\gamma$  production by T cells and natural killer cells, and appears to be necessary for the differentiation of Th1 type helper T cell responses (Robertson *et al.*, 1992; Manetti *et al.*, 1993). Moreover, IL-12 plays an important part in the activation and differentiation of cytotoxic T lymphocytes (CTL) (Gately *et al.*, 1992). It is this latter activity that is an important aspect of the anti-tumor immunity induced by IL-12 that has been demonstrated in animals (Brunda *et al.*, 1993). Previously our *in vitro* findings among patients with the Sézary form of CTCL indicated a profound deficiency in their ability to produce IL-12 (Rook *et al.*, 1993). It is also noteworthy that we demonstrated that recombinant IL-12 could normalize IFN- $\gamma$  production, enhance cell-mediated cytotoxicity and augment natural killer cell cytotoxicity when added to PBMC from advanced CTCL patients (Rook *et al.*, 1995). Previous clinical trials with recombinant human IL-12 (rhIL-12) for CTCL have demonstrated that it is a potent therapeutic agent, which induces cytotoxic T cell responses; however, repeated administration of IL-12 induced a reversible suppression of IL-12-dependent responses, such as IFN- $\gamma$  production, limiting the therapeutic effectiveness of the treatment. Although, the underlying mechanism of this refractoriness remains to be fully elucidated, observed downmodulation of IL-12 receptor (IL-12R) expression and increased degradation of STAT4 protein, a critical IL-12 signaling factor, provided plausible explanation for IL-12 induced unresponsiveness (Rook *et al.*, 1999; Wang *et al.*, 2001). Furthermore, studies of IL-12-treated rodents indicated that nitric oxide generated by macrophages played an important part in the suppression of IL-12 responses (Koblish *et al.*, 1998).

Different therapeutic regimens have been considered to improve the effectiveness of IL-12 in cancer therapy, including the combination of IL-12 with other biologic response modifiers such as IL-2. IL-2 is another potent Th1 type cytokine that is known for its anti-tumor activity and its augmentatory effects on cell-mediated immunity (Trinchieri *et al.*, 1984). IL-2 has also been shown to enhance the proliferation and cytotoxic function of natural killer cells *in vitro* and *in vivo* (Henney *et al.*, 1981; Trinchieri *et al.*, 1984; Caligiuri *et al.*, 1993). Importantly, IL-2 production was also found to be deficient by PBMC of Sézary patients (Rook *et al.*, 1993).

Although the combination of IL-12 and IL-2 has been associated with some toxicity in animal models, the treatment of murine tumors with IL-12 plus IL-2 was more effective than either of those cytokines alone in inducing tumor regression (Wigginton *et al.*, 1996). Recently, Wang *et al.* (2000), have shown that IL-2 can enhance the response of human natural killer cells to IL-12 through upregulation of IL-12R and increased phosphorylation of STAT4. These data suggest that combining IL-2 with IL-12 may result in augmented anti-tumor activity in humans.

In this study, we have adopted the use of combined biologic response modifiers as a more potent therapeutic approach to enhance the overall response rate of patients with CTCL and to overcome the refractoriness to rhIL-12 therapy observed in previous clinical trials. We studied the immunologic effects *in vitro* of adding IL-2 to IL-12 as a model to achieve these goals. The effect of these two potent cytokines on the stimulation of multiple immunologic parameters, including IFN- $\gamma$  production, enhancement of natural killer cell activity, and upregulation of IL-12R expression by T cells of CTCL patients were examined.

## MATERIALS AND METHODS

Patients participating in this study were diagnosed with Sézary syndrome (Edelson, 1980), the leukemic form of CTCL, on the basis of clinical, histopathologic, and immunohistologic criteria (Murphy, 1988). Characterization of patient circulating malignant T cells was performed

by analysis of 1  $\mu$ m sections of formalin-fixed peripheral blood buffy coats by detection of mononuclear cells possessing cerebriform nuclear morphology. In addition, fluorescence-activated cell sorting (FACS) analysis was used to select specifically typical malignant clonal populations that were CD4<sup>+</sup>/CD7<sup>-</sup> (Murphy, 1988). Using these two techniques, three groups of patients with Sézary syndrome were studied. The group, referred to as the high "tumor burden" group, consisted of four patients with Sézary counts ranging from approximately 50% up to 99% of mononuclear cells. The second group referred to as the intermediate "tumor burden" group consisted of five patients with Sézary counts that were approximately 20–50% of mononuclear cells, and the third group a low "tumor burden" group consisted of six patients with Sézary counts ranging from approximately 10 to 20% of mononuclear cells. None of the patients were receiving cytotoxic drugs or systemic or topical corticosteroids at the time of study. Samples from 13 healthy volunteers served as controls. Donations of blood by patients (n = 15) or normal volunteers (n = 15) in this study conformed to IRB-approved protocol and informed consents were obtained.

**Cytokines, antibodies, and reagents** Mouse IgG1 antibodies against human IL-12R  $\beta$ 2 chains were a generous gift from Dr Kathy Wang and Dr Jerome Ritz (Dana-Farber Cancer Institute, Boston, MA). Phycoerythrin-labeled goat anti-mouse antibodies were purchased from Jackson Immuno-Research Laboratories, Inc. (Jennersville, PA). Mouse IgG1 isotype control, rat IgG and goat serum blocking reagents were purchased from Sigma (St Louis, MO). Fluorescein isothiocyanate- and peridinin chlorophyll protein (PerCP)-labeled antibodies for CD4<sup>+</sup> and CD8<sup>+</sup> cells were purchased from Pharmingen (San Diego, CA) and Becton Dickinson (San Jose, CA), respectively. rIL-12 was provided by Dr Stanley Wolf of the Genetics Institute (Andover, MA) and rhIL-2 was purchased from Boehringer Mannheim (Indianapolis, IN).

**PBMC preparation and culture criteria** PBMC were prepared as described previously (Rook *et al.*, 1995). Briefly, venous blood was collected into heparinized syringes using uniform standards for both Sézary syndrome patients and normal volunteers. The blood was then diluted 2-fold with Dulbecco's phosphate-buffered saline (BioWhittaker, Walkersville, MD), pH 7.2, layered over Ficoll-Hypaque (Amersham, Uppsala, Sweden), and centrifuged at 500  $\times$  g for 30 min at room temperature. The interface containing the mononuclear cell fraction was collected and cells were washed three times with phosphate-buffered saline. Cells were used immediately after purification.

Cell cultures were set up at a final concentration of  $1 \times 10^6$  cells per ml in RPMI 1640 media (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 200 mM per l L-glutamine, and 100 U penicillin-streptomycin per ml for 70 h. Certain cultures were supplemented with the mitogen phytohemagglutinin (PHA) (1  $\mu$ g per ml or 2  $\mu$ g per ml), predetermined concentrations of cytokines (IL-12: 1 ng per ml; and IL-2: 100 U per ml).

**Cell surface staining and flow cytometric analysis of IL-12R  $\beta$ 2 subunits on T subset CD4<sup>+</sup> and CD8<sup>+</sup> cells** Analysis of the expression of IL-12R  $\beta$ 2 subunits on CD4<sup>+</sup> and CD8<sup>+</sup> cells was accomplished using flow cytometric analysis. Approximately  $10^6$  PBMC per sample were harvested from different cell cultures and washed twice with washing solution (1  $\times$  phosphate-buffered saline, 3% fetal bovine serum, pH 7.4). Cells were then incubated with Rat IgG (Sigma) for 10 min on ice to reduce nonspecific binding. Mouse IgG1 against IL-12R  $\beta$ 2 (Dana-Farber Cancer Institute, Boston, MA) subunit at concentrations of 10  $\mu$ l of 1:1000 dilution of acetic fluid per 50  $\mu$ l phosphate-buffered saline containing 3% fetal bovine serum, was then added to the cells and incubated at 4°C for 30 min. Purified mouse IgG1 (Sigma) at the same concentration was used as an appropriate control antibody. Cells were subsequently washed twice with washing media and incubated with 20  $\mu$ l goat serum (Sigma) on ice for 10 min to block nonspecific binding. Finally phycoerythrin-labeled goat anti-mouse IgG1 secondary antibody (BioSource International, Camarillo, CA) was added at a concentration of 1:50 dilution.

To differentiate the expression of IL-12R  $\beta$ 2 on CD4<sup>+</sup> and CD8<sup>+</sup> subpopulation, cells were stained with fluorescein isothiocyanate-labeled anti-CD4 (Pharmingen) and PerCP-labeled anti-CD8 (Becton Dickinson) antibodies. Fluorescein isothiocyanate- and PerCP-labeled mouse IgG were used as controls, respectively. After washing, stained cells were directly analyzed on a FACScan flow cytometer (Becton Dickinson). Data were processed by using the CELLQuest program (Becton Dickinson). Typically 10,000 cells in each sample were analyzed. The level of IL-12R  $\beta$ 2 subunit expression was measured by gating on CD4<sup>+</sup> or CD8<sup>+</sup> cells and was expressed as percentage positive cells expressing the IL-12R  $\beta$ 2.

**Cytokine assays** IFN- $\gamma$  was assayed by colorimetric enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) according to the manufacturer's specifications as previously described (Rook *et al*, 1995).

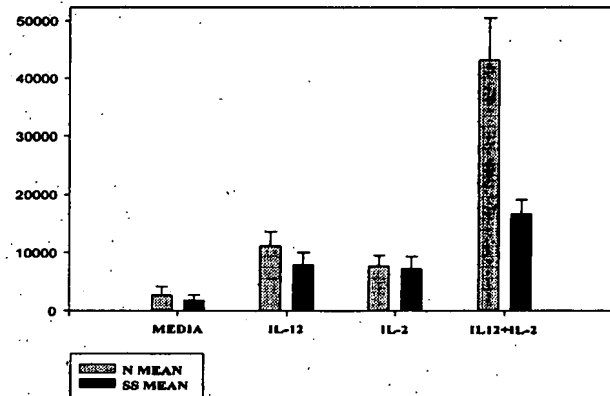
**Natural killer cell function** PBL were separated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation. Cells were washed three times and were resuspended in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum. Target cells that were used in assays for natural killer cell function were prepared from suspension cultures of the K562 line. The assay was performed as previously published (Rook *et al*, 1995).

**Statistical analysis** The data on IFN- $\gamma$  production were analyzed using an unpaired, two-tailed Student's *t* test or the Wilcoxon ranked sum test for paired groups to determine the statistical significance of the data, and *p* < 0.05 was considered significant.

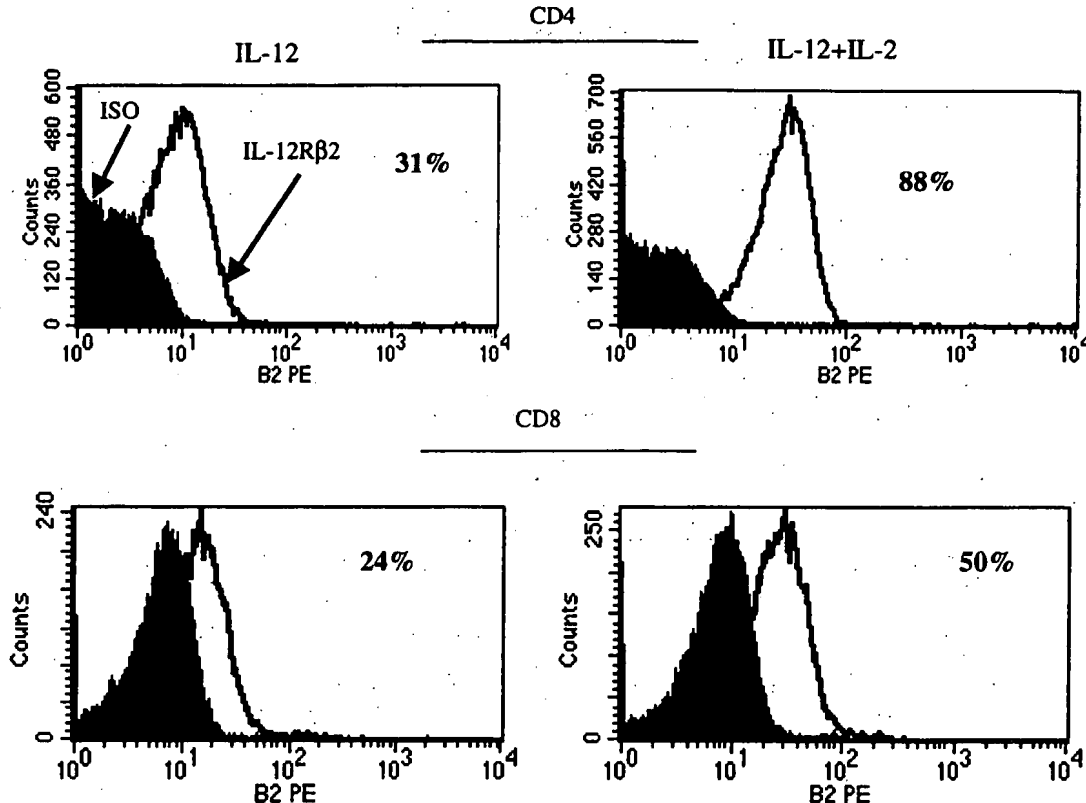
## RESULTS

**IL-12 plus IL-2 synergistically enhance the production of IFN- $\gamma$  by PBMC from Sézary syndrome patients** Previous studies have demonstrated that recombinant IL-12 can enhance IFN- $\gamma$  production by the PBMC of patients with Sézary syndrome (Rook *et al*, 1995). Nevertheless, during treatment of patients with IL-12, refractoriness to this biologic effect frequently occurs in association with clinical refractoriness to IL-12 (Gollob *et al*, 1998; Rook *et al*, 1999). As a model for overcoming the refractory state, we examined the immunologic effects of adding IL-2 to IL-12. IFN- $\gamma$  production was examined using the PBMC of 15 patients with Sézary syndrome with varying burdens of circulating malignant T cells as described in *Materials and Methods*, and PBMC from 15 healthy sex- and age-matched volunteers as controls. Mononuclear cells were cultured with either 1 ng IL-12

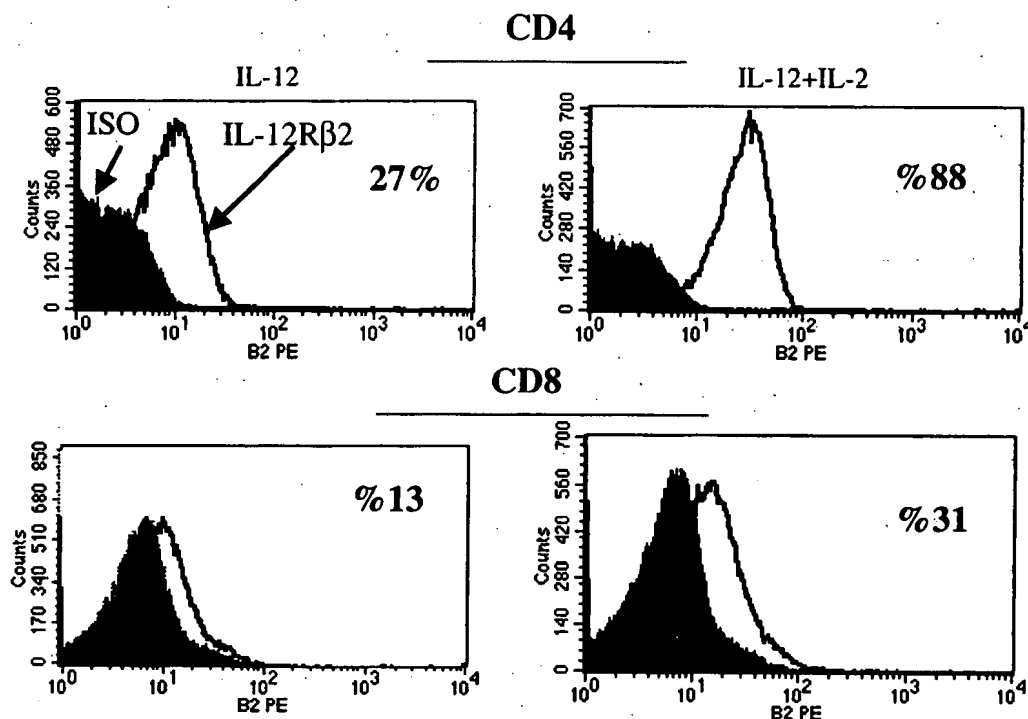
per ml or 100 units of IL-2 per ml or both with 1  $\mu$ g PHA per ml for 48 h. Supernatants were assayed for IFN- $\gamma$  levels by enzyme-linked immunosorbent assay. As shown in Fig 1, IL-12 induced substantial IFN- $\gamma$  production by the patients' PBMC (mean  $\pm$  SE 7914 pg per ml  $\pm$  2161) as did IL-2 alone (mean 7222 pg per



**Figure 1. Synergistic effect of IL-12 plus IL-2 on IFN- $\gamma$  production by PBMC from Sézary syndrome patients.** PBMC stimulated with PHA were cultured with either IL-12, IL-2, or IL-12 plus IL-2 for 70 h. Supernatants were assayed for the presence of IFN- $\gamma$  by enzyme-linked immunosorbent assay. Light gray bars represent values for normal volunteers, whereas dark gray bars represent values for Sézary syndrome patients.



**Figure 2. IL-12 plus IL-2 increase the expression of IL-12R  $\beta$ 2 on T cells from normal volunteers.** PBMC from normal volunteers were cultured in the presence of PHA and either IL-12, IL-2, or IL-12 plus IL-2 for 70 h. Analysis was performed by flow cytometry after gating on CD4 $^{+}$  and CD8 $^{+}$  T cells. Data represent percentage of cells expressing IL-12R  $\beta$ 2. A representative experiment is shown using the PBMC of a normal volunteer.



### Low Tumor Burden Patient

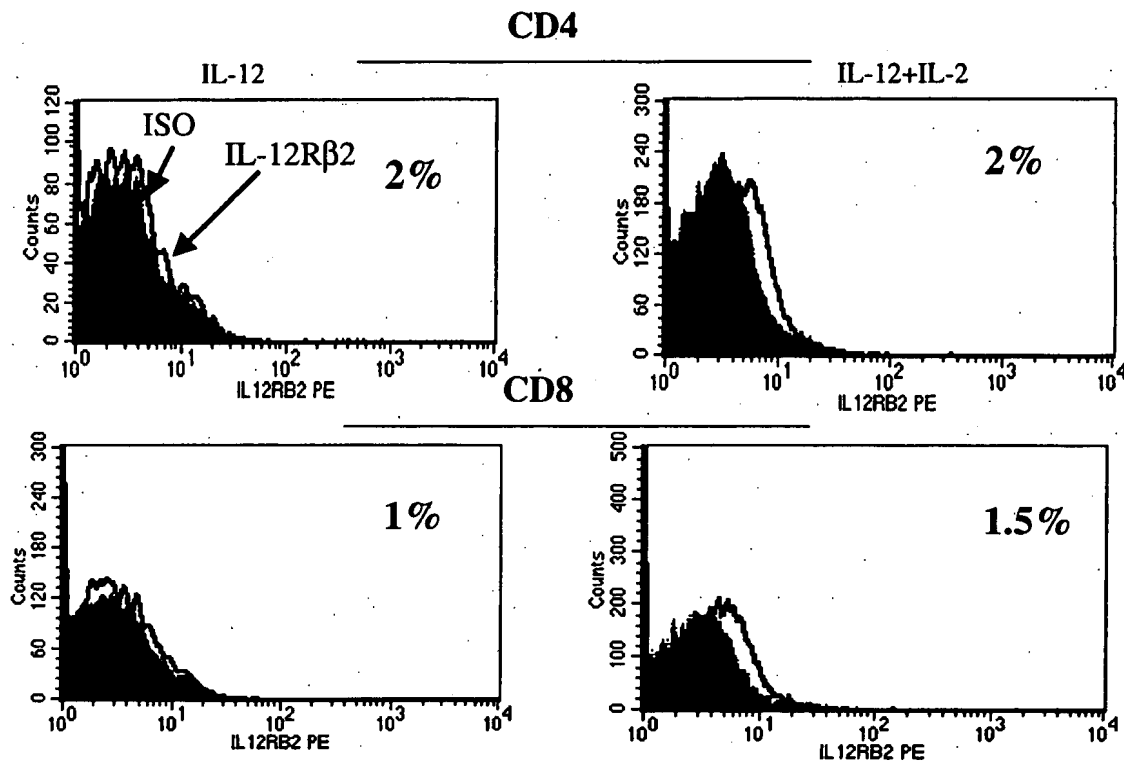
**Figure 3.** IL-12 plus IL-2 increase the expression of IL-12R  $\beta$ 2 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells of low tumor burden Sézary syndrome patients. PBMC from a Sézary syndrome patient with a low circulating tumor burden were cultured in the presence of PHA and were stimulated with IL-12, IL-2, or both for 70 h. Analysis was done by flow cytometry after gating on CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Similar results were obtained using the cells of five more patients with a low burden of circulating malignant T cells.

ml  $\pm$  2228). It is noteworthy that addition of IL-2 to IL-12 markedly enhanced the IFN- $\gamma$  produced by the PBMC of all patients studied in comparison with IL-12 or IL-2 alone (mean 16790 pg per ml  $\pm$  2490) ( $p < 0.01$ ).

**Addition of IL-2 to IL-12 markedly upmodulates the IL-12R  $\beta$ 2 expression on T cells of Sézary syndrome patients** The IL-12R  $\beta$ 2 is the signaling receptor through which IL-12 transduces its signal to IL-12 responsive genes (Presky *et al*, 1996). Therefore, in an effort to understand the potential synergistic effects of adding IL-2 to IL-12, the ability of these cytokines to modulate IL-12R  $\beta$ 2 expression on T cells of Sézary syndrome patients was examined. Freshly isolated T cells from Sézary syndrome patients and age- and sex-matched normal volunteers were cultured with either a suboptimal dose of PHA alone (1–2  $\mu$ g per ml) or with combinations of 1 ng IL-12 per ml, 100 units of IL-2 per ml, or both with PHA for 70 h followed by flow cytometric analysis. IL-12 plus IL-2 induced more expression of IL-12R  $\beta$ 2 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells from healthy controls in comparison with IL-12 alone. A representative individual experiment is shown in Fig 2. T cells from three groups of patients with Sézary syndrome were also studied for the modulatory effects of IL-2 added to IL-12. These groups included those with high, intermediate, or low circulating tumor burden as described in *Materials and Methods*. PBMC from patients with a low circulating burden of malignant T cells demonstrated a marked enhancement of IL-12R  $\beta$ 2 expression when cells were cultured with IL-2 plus IL-12 in comparison with IL-12 alone (Fig 3). For example, after culture of one Sézary patient's PBMC with IL-2 plus IL-12, 88% of CD4<sup>+</sup> T cells and 31% of CD8<sup>+</sup> T cells expressed IL-12R  $\beta$ 2 as compared with only 27% of CD4<sup>+</sup> T cells and 13% of CD8<sup>+</sup> T cells after culture with IL-12 alone (Fig 3). In contrast to results with

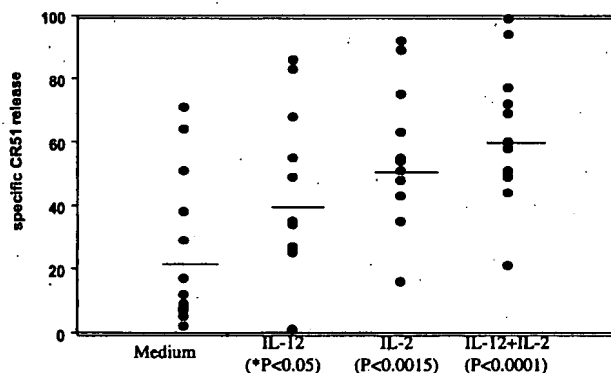
low tumor burden patients, those with an intermediate and high burden of circulating malignant T cells demonstrated progressively diminished responses of IL-12R  $\beta$ 2 expression when their PBMC were cultured with IL-12 alone or with IL-2 in addition to IL-12 (Fig 4).

**The functional consequences of adding IL-2 to IL-12: Enhanced natural killer cell activity of Sézary syndrome patients** Although IL-2 plus IL-12 appear to enhance the production of IFN- $\gamma$  by the cells of all patients with Sézary syndrome and to upmodulate the expression of IL-12R  $\beta$ 2 on the PBMC of many patients, the functional consequences of these cytokines to increase cell-mediated immunity may be most clinically relevant. Therefore, one measure of cell-mediated immunity that we chose to study was the modulation of natural killer cell activity. The PBMC of 15 patients with Sézary syndrome were cultured with either medium alone, 1 ng IL-12 per ml, 100 units IL-2 per ml or both cytokines for 24 h followed by a  $^{51}\text{Cr}$  release assay using K562 cells as target cells as previously described (Rook *et al*, 1995). As shown in Fig 5, significant augmentation of natural killer cell activity occurred with either IL-12 ( $p < 0.5$ ) or IL-2 ( $p < 0.0015$ ) alone in comparison with medium and the mean natural killer cell activity of the 15 patients was further boosted by combining IL-12 with IL-2 ( $p < 0.0001$ ). It is noteworthy that even some patients with very high levels of circulating malignant T cells experienced significant additional augmentation of natural killer cell activity when their PBMC were cultured with IL-12 plus IL-2 in comparison with either cytokine alone or to medium alone. An example of this effect is shown in Fig 6 where an experiment was performed using the PBMC of a patient who had 90% of their circulating mononuclear cells identified as Sézary cells by morphologic and by flow cytometric criteria.

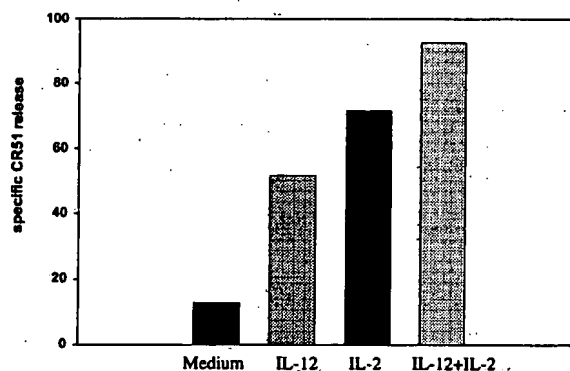


### High Tumor Burden patient

**Figure 4.** IL-12 plus IL-2 fails to upregulate the expression of IL-12R  $\beta$ 2 on T cells of Sézary syndrome patients with a high circulating burden of malignant T cells. PBMC from a Sézary syndrome patient with a high circulating tumor burden were cultured in the presence of PHA and were stimulated with IL-12, IL-2, or both for 70 h. Analysis was done by flow cytometry after gating on CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Similar results were obtained using the cells of three more patients with a high burden of circulating malignant T cells.



**Figure 5.** IL-12 plus IL-2 augment natural killer cell activity of Sézary syndrome patients. PBMC from patients with Sézary syndrome ( $n = 15$ ) were cultured for 24 h with either medium, IL-12, IL-2, or IL-12 plus IL-2 followed by a 4 h Cr<sup>51</sup> release assay using K562 cells as targets. The effector to target ratio shown is 25:1.



**Figure 6.** IL-12 plus IL-2 augments natural killer cell activity of a Sézary syndrome patient with a high burden of circulating malignant T cells. PBMC from a patient with 90% circulating malignant T cells were cultured for 24 h with either medium alone, IL-12 alone, IL-2 alone, or IL-2 plus IL-12 followed by a 4 h Cr<sup>51</sup> release assay using K562 cells as targets. The effector to target ratio shown is 25:1.

### DISCUSSION

Two previous clinical trials using recombinant IL-12 to treat CTCL indicated that this cytokine is biologically active for this disorder with the demonstration of approximately a 50% response rate characterized by significant clearing of skin lesions (Rook *et al*, 1999; Gollob *et al*, 2000). Nevertheless, clinical refractoriness, as evidenced by the early relapse of patients who had experienced a

complete response or by the early plateau of a partial response, was not an infrequent observation during both clinical trials. Our preliminary findings have related this clinical phenomenon to decreased IFN- $\gamma$  production and to the downmodulation of IL-12R  $\beta$ 2 expression (AH Rook *et al*, unpublished observations). Similarly, Gollob *et al* (2000) observed a clinical phenotype among IL-12 responsive solid tumor patients that correlated with the persistent induction of IFN- $\gamma$ , IL-15, and IL-18 following the long-

term administration of IL-12. Conversely, patients who were clinically unresponsive to IL-12 exhibited a rapidly diminished capacity to produce IFN- $\gamma$  and IL-15 during IL-12 administration. Thus, in an effort to overcome the clinical and biologic refractoriness to IL-12 and to enhance the clinical benefit of IL-12 treatment for CTCL patients, we postulated that addition of another IFN- $\gamma$  inducing cytokine to IL-12 could potentially have salutary therapeutic effects.

Because IL-2 is known to induce potently IFN- $\gamma$  production independently of IL-12, it was a logical choice of cytokines to combine with IL-12 (Chan *et al*, 1991). The results of our study clearly establish that combining IL-2 with IL-12 leads to the synergistic enhancement of IFN- $\gamma$  production by PBMC derived from patients with Sézary syndrome. In fact, regardless of the circulating burden of malignant T cells, the PBMC of all patients studied manifested this immune potentiating effect of combining IL-2 with IL-12. Thus, it is anticipated that adding IL-2 to rIL-12 therapy might at least partially prevent the attenuation of IFN- $\gamma$  production that occurs during the chronic use of rIL-12.

Previous studies have identified IFN- $\gamma$  as a potent upmodulatory cytokine for expression of the IL-12R  $\beta$ 2 (Zaki *et al*, 2001). In these studies, IFN- $\gamma$  appeared to induce the most significant expression of IL-12R  $\beta$ 2 on the T cells of Sézary syndrome patients in comparison with IFN- $\alpha$ , IL-18, and IL-12 itself (Zaki *et al*, 2001). Furthermore, patients who had a more modest circulating burden of tumor cells had T cells that were more responsive to IFN- $\gamma$  in regard to IL-12R expression in comparison with patients with a high circulating tumor burden. The results of this study are consistent with these previous findings in that patients with a more modest circulating tumor burden exhibited a more pronounced augmentation of IL-12R  $\beta$ 2 expression in response to IL-12 and IL-2 in comparison with patients with a high circulating tumor burden. It remains to be established what the underlying mechanisms are that are responsible for the low IL-12R  $\beta$ 2 expression on the T cells of these patients and to what extent the signaling pathways in their remaining normal T cells are impaired. Our findings demonstrate a discrepancy between the levels of IL-12R  $\beta$ 2 expression on the T cells of heavy tumor burden patients and the ability of their cells to produce increased levels of IFN- $\gamma$  in response to IL-12 plus IL-2.

Although the effects of combined IL-12 plus IL-2 on IL-12R  $\beta$ 2 on natural killer cells of CTCL patients have not been examined in recent studies, Wang *et al* (2000) recently reported that the combination of these cytokines elevated the expression of IL-12R on natural killer cells and enhanced IL-12 signaling in natural killer cells. It is therefore possible that natural killer cells from our patients, including those derived from heavy tumor burden patients were important participants in the IFN- $\gamma$  production process. Moreover, these results with natural killer cell enhancement indicate that individual patients with advanced disease can respond with synergistic increases in natural killer cell activity. Two patients with more than 90% circulating malignant T cells who demonstrated such an enhancement of natural killer cell activity in response to IL-2 plus IL-12 were an excellent example of this phenomenon. As natural killer cell activity may be an excellent surrogate marker for the functional establishment of cytolytic T lymphocyte activity, our findings are clearly relevant to the generation of host anti-tumor activity. The importance of cytolytic T lymphocytes in CTCL has been supported by the findings of Hoppe *et al* (1995), demonstrating a correlation between the presence of CD8<sup>+</sup> tumor infiltrating lymphocytes in skin lesions and favorable prognosis among patients. Furthermore, our observations among IL-12-treated CTCL patients indicate that IL-12 potently induces cytolytic T lymphocyte infiltrates within regressing skin lesions (Rook *et al*, 1999). Our preliminary studies demonstrate the capacity of these infiltrating CD8<sup>+</sup> T cells to mediate apoptosis of the CD4<sup>+</sup> malignant T cells (LE French and AH Rook, unpublished observations). Thus, the results of this study provide reason to be optimistic regarding a future therapeutic approach utilizing IL-2 plus rIL-12 for patients with cutaneous T cell lymphoma.

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## Imiquimod Applied Topically: A Novel Immune Response Modifier

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### ABSTRACT

*Imiquimod (S-26308, R-837) (1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4 amine), an immune response modifier, was approved as a 5% cream (Aldara, 3M Pharmaceuticals) by the US FDA in February 1997, for the treatment of genital and perianal warts. Drug activity results primarily from induction of interferon alpha (IFN- $\alpha$ ) and other cytokines in the skin, which stimulate several other aspects of the innate immune response. Imiquimod also stimulates acquired immunity, in particular the cellular arm, which is important for control of viral infections and tumors. It is expected to be effective where exogenous IFN- $\alpha$  has shown utility, and where enhancement of cell-mediated immunity is needed. Recently presented Phase II clinical studies demonstrated efficacy in treating UV induced skin lesions, basal cell carcinoma, and actinic keratosis. Case studies have reported benefit when treating molluscum and in prevention of keloids after surgery.*

**KEY WORDS:** imiquimod, immune response modifier, warts

The Th1 CMI response is very effective in most people for controlling viral infections and tumors. For example, chicken pox is almost universal and after an outbreak, the varicella zoster virus responsible is carried in the dorsal root ganglia for the rest of the individual's life. Usually, no further lesions occur, except in those 20% of individuals who eventually develop herpes zoster. In another example, epidemiology studies report the Human Papillomavirus (HPV) is a frequently occurring infection with 50-75% of sexually active adults having an antibody response to the virus.<sup>1</sup> About 15% of these individuals carry the virus and a severe outbreak of warts can occur if the cellular immune response is suppressed because of anti-graft rejection drugs following transplantation, anti-cancer chemotherapy, acquiring HIV infection, or in some cases, pregnancy.

Genital warts, the most common viral sexually transmitted disease, was chosen as the first clinical target for imiquimod because injectable interferon alpha (IFN- $\alpha$ ) had demonstrated some benefit, and current therapies had not met the physicians' or patients' needs. Patient dissatisfaction was significant due to pain, tissue destruction, high recurrence rates, expense, and the time required for treatment. As well, current treatments only targeted the visible warts and did not treat the underlying HPV infection.

Published results indicate that biopsies from these patients' warts showed little immune recognition, but biopsies from warts undergoing spontaneous regression showed monocytic cellular infiltration and increased Th1 cytokine expression.<sup>2,3</sup> Similar results were seen in patients treated with interferon.<sup>4</sup>

### Mechanism of Action

The exact biochemical mechanism of action for imiquimod is not known. However, studies have reported the following evidence:

- In human peripheral blood mononuclear cells (PBMCs), specifically monocytes, imiquimod at a low concentration of 1-5  $\mu$ g/ml induces cytokine production including several subtypes of IFN- $\alpha$ , TNF- $\alpha$ , IL-1, IL-1RA, IL-6, IL-8, IL-10, IL-12 p40, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 1- $\alpha$  (MIP-1 $\alpha$ ), MIP-1 $\beta$ , and macrophage chemotactic protein (MCP-1).<sup>5,6</sup>
- Topical application of the 1% or 5% cream formulation of imiquimod to the skin of hairless mice increases IFN- $\alpha$  messenger RNA (mRNA) levels, and higher protein concentrations of IFN and TNF- $\alpha$  in the skin at the treatment site.<sup>7,8</sup>

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- Topical treatment of hairless mice with imiquimod causes Langerhans cells in the skin to enlarge, appear activated and migrate from the treatment site to the regional lymph node.<sup>9</sup> These activated cells may enhance antigen presentation to T-cells.
- Imiquimod was shown to be effective in animal models against a number of viral infections and a variety of transplantable tumors.<sup>10,11</sup> The duration of antiviral activity lasts for 3-4 days after each oral imiquimod administration and correlates with elevation of oligoadenylate synthetase (2',5'-AS) activity, though this increase is indirect through IFN- $\alpha$  production.<sup>12</sup> Elevated 2',5'-AS was observed in the serum of mice, rats, guinea pigs, monkeys, and humans<sup>7</sup> from 24-72 hours after oral treatment.
- Antitumor activity of imiquimod is also seen in a number of transplantable mouse tumor models.<sup>13</sup> Much of this antitumor effect is blocked by administration of antibodies to IFN- $\alpha$ .
- Imiquimod was effective at inhibiting growth of the human mammary tumor MCF-7 when transplanted into nude mice lacking T-cells, indicating that acutely, T-cells are not required for its antitumor effects.<sup>13</sup>
- Although it does not stimulate T-cells to divide, nor does it directly induce T-cell cytokines such as IL-2, IL-4 or IL-5, imiquimod can indirectly stimulate production of the T helper type 1 (Th1) cytokine, IFN- $\gamma$ , in mouse splenic and bone marrow cultures, as well as human PBMC cultures. Production of IFN- $\gamma$  in response to imiquimod is inhibited by antibodies to IL-12 and IFN- $\alpha$ , demonstrating the importance of these monocyte/macrophage cytokines.<sup>14</sup> The mechanism of interaction between these cytokines has recently been defined.<sup>15,16</sup> Results show that IFN- $\alpha$  induces the IL-12 receptor  $\beta$ 2 subunit on Th1 cells. These cells can then respond to IL-12 and produce IFN- $\gamma$ . Thus, Th1 cells are the major source of IFN- $\gamma$ . However, cytotoxic T-cells and natural killer (NK) cells are also able to produce IFN- $\gamma$  in response to imiquimod.
- Imiquimod was reported to inhibit production of the Th2 cytokine IL-5 in both mouse and human cell systems, and as a result, it has also been found to inhibit both antigen and *Sephadex* induced eosinophilia in several animal models.<sup>17</sup>
- Results from a Phase I double-blind, randomized, parallel group study done in humans showed that all imiquimod treated patients had a  $\geq 75\%$  reduction in wart area.<sup>18</sup> Imiquimod treatment stimulated significant increases in IFN- $\alpha$  as well as increases in TNF- $\alpha$  mRNAs, cytokines previously found to be induced by imiquimod in animal studies<sup>7,10,19,20</sup> and in human PBMC studies.<sup>5,6</sup>

Imiquimod may be useful in atopic diseases as well as other diseases where an increased Th1 response is needed. Wart regression by imiquimod is associated with an induction of local

cytokines and cellular infiltrates that are involved with the generation of a cell mediated immune response.

### *Summary of Clinical Efficacy Trials*

A Phase II study of 108 patients with genital warts compared topically applied 5% imiquimod cream to vehicle cream (see Table 1).<sup>21</sup> The imiquimod group had 40% "complete wart clearance" compared to no "complete clearance" in the vehicle group. In addition, there was a median 90% reduction in wart area at the end of treatment among the imiquimod group, but no change in wart area in the vehicle treated group. Patients with totally cleared lesions entered a 10-week follow up period to observe wart recurrence and 81% of the imiquimod treated group remained wart free.

A Phase III multi-centered, randomized, double blind, placebo controlled trial compared the safety and efficacy of imiquimod 5% cream, 1% cream and vehicle.<sup>22</sup> The main outcome measurements were the number of patients experiencing complete elimination of all baseline warts and wart recurrence. The reduction in baseline wart area, the duration of therapy required to eliminate warts, and the frequency and severity of adverse reactions were also monitored. Patients with total wart clearance entered into a 12-week follow up to monitor recurrence. The difference between the effectiveness of the 5% cream and vehicle was statistically significant ( $p < 0.0001$ ). The results using 1% cream were not significantly different from vehicle. The median time to clearance was 10 weeks, 12 weeks, and 12 weeks, respectively. Females had a higher clearance rate (77%, 46%, and 28%, respectively) than males (40%, 10%, and 6%, respectively), and females had a shorter median time to clearance (8 weeks) than males (12 weeks) in both imiquimod groups. The better response in females could be due to several factors including shorter duration of warts in females (3.4 months median) vs males (6.7 months median), better compliance for females, or better drug absorption in females. The treatment was well tolerated. Local erythema was the most common adverse reaction (67%, 26%, and 24%, respectively) but the majority of patients experienced no or only mild local inflammatory reactions.

In a second Phase III trial the difference between the effectiveness of the 5% cream and vehicle was also statistically significant ( $p < 0.0001$ ), though clearance in the 1% vs the vehicle group was not. Recurrence rates were 19% (9/48) for 5% imiquimod group, 17% (2/12) for the 1% group, and 0% (0/3) for the vehicle group. The low recurrence rate in the vehicle groups is not surprising since the mechanism of spontaneous clearance was shown to be due to immune recognition.<sup>23</sup> Local skin reactions were more common and more severe with daily treatment, but there were no systemic adverse reactions. Results were again better in women. Both 3 times/week and daily treatment regimens were acceptable for safety and efficacy, however in the final analysis, most patients preferred the 3 times/week regimen.<sup>23</sup>



			Application	% Complete Wart Clearance	Recurrence Rate	Adverse Reactions
Beutner, et al (1998 Feb) <sup>21</sup>	Phase II	108 patients with genital warts	23-24 hour application 3 days/wk for 8 wks	5% Imiquimod Cream: 40%	19%	
				Vehicle Cream: 0%	N/A	
Edwards, et al (1998) <sup>22</sup>	Phase III, multicenter, randomized, double-blind, placebo controlled	180 males 131 females ≥ 18 yrs old with 2-50 external anogenital warts	8 hour application overnight, 3x/wk for 16 wks or until totally clear	5% Imiquimod Cream: 50%	13%	Local erythema; no systemic adverse reactions
				1% Imiquimod Cream: 21%	0%	
				Vehicle Cream: 11%	10%	
Beutner, et al (1998 Apr) <sup>23</sup>	Phase III, multicenter, randomized, double-blind, placebo controlled	154 males 125 females with genital warts	8 hour application overnight daily for 16 wks or until totally cleared	5% Imiquimod Cream: 71%	19%	Local erythema; no systemic adverse reactions
				1% Imiquimod Cream: 16%	17%	
				Vehicle Cream: 4%	0%	
Conant, et al (1998) <sup>24</sup>	Multicenter, double-blind, vehicle controlled, parallel group trial	97 males 3 females who are HIV positive and have genital warts	Treated 3x/wk for 16 wks or until totally cleared	5% Imiquimod Cream: 11% (>50% wart reduction: 38%)		Mild erythema
				Vehicle Cream: 6% (>50% wart reduction: 14%)		

Table 1: A review of clinical efficacy trials.

A vehicle controlled safety and efficacy trial was done in HIV-positive genital wart patients.<sup>24</sup> The primary objective of this trial was to evaluate the safety of imiquimod 5% cream in HIV-positive patients. A secondary objective was to assess wart clearance and reduction in the wart area. No local skin reactions were seen in a majority of patients and only mild erythema was seen in most of the others. The difference between the effectiveness of the 5% cream and vehicle was not significantly different. However, there was a statistically significant difference between treatment groups for patients who achieved >50% reduction in wart area, 38% for imiquimod, and 14% for vehicle ( $p=0.013$ ). This was a clinically meaningful reduction in wart area since wart area increases are frequently seen in these patients. These results suggest that in HIV patients, imiquimod induces the innate response that stops wart growth and causes wart area reduction and may, in part, be IFN- $\alpha$  mediated. However, the reduced total wart clearance in HIV patients compared to immunocompetent genital wart patients suggests a role for T-cell responses in initial wart clearance as well as in long term protection from recurrence. Imiquimod has an acceptable safety profile in HIV-positive and AIDS patients.

Imiquimod's mechanism of action should also be effective for treating other chronic virus skin infections such as common warts, plantar warts, herpes simplex virus infection, and molluscum contagiosum, as well as skin tumors. Small studies have reported success in treating molluscum,<sup>25,26</sup> and a case report shows treatment of a patient with recalcitrant facial flat warts.<sup>27</sup> Results of a small pilot trial of imiquimod 5% cream in patients with Bowen's disease showed that 14 of 16 patients cleared their lesions.<sup>28</sup> A pilot study showed success in treating basal cell carcinoma,<sup>29</sup> and Phase II results were recently reported (Geisse, personal communication, 2000 Oct). Phase II results were also reported for actinic keratosis (Stockfleth, personal communication, 2000 Oct). Other skin tumors that might respond include Kaposi's sarcoma and cutaneous T-cell lymphoma since they have been reported to respond to interferon therapy.<sup>30,31</sup>

Since imiquimod inhibits Th2 responses in preclinical animal models, atopic based skin inflammation such as atopic dermatitis might also benefit. Other conditions that have responded to topically applied imiquimod include alopecia areata (Stockfleth, personal communication, 2000 Oct) and keloids (Berman, personal communication, 2000 Oct).

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Another possible use for these drugs is application with a vaccine for adjuvant activity. The imidazoquinolines are expected to enhance a Th1 response to the vaccine, which could be beneficial for virus or tumor vaccines. Drug application topically or transdermally could be explored with the injectable vaccine. On the other hand, skin inflammation due to excessive Th1 responses, such as psoriasis and contact dermatitis, might be worsened by topical treatment with imiquimod. Imiquimod is unique in being a topically active cytokine inducer and stimulant for the CMI response.

## Conclusion

Wart regression by imiquimod is associated with an induction of local cytokines and cellular infiltrates that are involved with the generation of a cell mediated immune response. These results in humans are consistent with the preclinical results reported in animal models. Overall, imiquimod applied topically is an immune response modifier that would be a useful addition to the drugs that can be used to treat significant chronic conditions of the skin. As such, imiquimod applied topically, represents a new class of drug.

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## ENHANCEMENT OF MIXED LEUKOCYTE REACTION AND CYTOTOXIC ANTITUMOR RESPONSES BY HEPARIN<sup>1</sup>

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The immunomodulating effects of heparin and natural and synthetic heparinoids (which are now undergoing clinical trials for the treatment of AIDS) on cellular immunity (DNA synthesis and cytotoxic responses of mouse lymphocytes to allogeneic cells and histocompatible tumors) were studied. The results showed that (1) high and low m.w. heparin enhanced mouse antitumor and antiallogeneic cell responses *in vitro*; (2) other sulfated heparinoids did not have this enhancing activity and some of them (including dextran sulfate) totally suppressed generation of cytotoxic cells; (3) these immunomodulating activities of heparin and heparinoids did not correlate with their anticoagulant effects, degree of sulfation, and mitogenic activity; (4) heparin did not increase the production of IL-2 and did not enhance the action of IL-2 on the cells in MLC, heparin also had no effect on the growth-promoting activity of IL-2 on cloned cytotoxic T cells; (5) heparin had a synergistic enhancing effect with IL-1 on the generation of cytotoxic cells in MLC; and (6) heparin abolished endothelial cell growth factor-induced suppression of cytotoxic response. The latter two effects by themselves, however, could not fully explain the entire immunoenhancing activity of heparin. These results indicate that heparin and heparinoids have multiple effects on the immune system and that some of them can enhance, whereas others can suppress cell-mediated responses.

Heparin is an acidic glycosaminoglycan of 12,000 to 20,000 m.w., composed of sulfated glucosamine and uronic acid residues (1, 2). *In vivo*, heparin is biosynthesized as a proteoglycan and is normally present in basophilic granules of mast cells and leukocytes, from which it is released during inflammatory reactions (2). Related heparan sulfate, chondroitin sulfates, dermatan sulfate, and keratan sulfate proteoglycans are found intercalated in the cell membranes of various cells, including endothelial cells, and are also present in extracellular matrix and basement membranes (2, 3).

More than 900 billion units (6 metric tons) of commercial (porcine) heparin is used in the United States per year for anticoagulant therapy and prophylaxis on approximately 10 million patients (1). Besides its anticoag-

ulant activity, however, heparin has numerous other biologic effects, mainly due to its ability to interact with basic plasma and cell surface components. Heparin binds to antithrombin, thrombin, low- and very low-density lipoproteins, platelet factor 4, fibronectin, fibrinogen, plasmin, protamine, complement components, several growth factors, and inositol triphosphate receptor (1, 2, 4-7). Heparin has also substantial antitumor activity in animals (8-10). However, potential physiologic role and therapeutic or pathologic significance of these interactions of heparin is still poorly understood.

Recently heparin and heparinoids (such as dextran sulfate) were found to inhibit infection of human T lymphocytes by the HIV virus (11-14), which prompted initiation of clinical trials of heparinoids for the treatment of AIDS. Very little is known, however, about the effects of heparin on the functions of lymphocytes and systematic evaluation of immunomodulating effects of heparin and heparinoids is urgently needed. In a few studies, mostly inhibitory effects of heparin were observed on the delayed type hypersensitivity (15), on allogeneic MLR (16), on mitogen- or Ag-induced blastogenesis (17), on cytotoxicity of NK cells (when heparin was present during the cytotoxicity assay) (18), and at very high concentrations (300 to 20,000 U/ml of heparin) on the eosinophil-mediated killing of *Trypanosoma cruzi* (19). Recently, enhancement of mitogenic responses of human lymphocytes by heparin was reported (20).

We report that heparin markedly enhances the proliferative and cytotoxic responses in murine allogeneic MLR and cytotoxic responses to histocompatible tumors. We have also compared the immunomodulating activities of heparin and several natural and synthetic heparinoids and evaluated the effect of heparin on the responses of lymphoid cells to IL-1, IL-2, and heparin-binding ECGF<sup>2</sup>.

### MATERIALS AND METHODS

**Lymphocytes and cell lines.** Spleen lymphocytes, depleted of E by hypotonic shock, were obtained from male or female C57BL/6 (H-2<sup>b</sup>), CBA/J (H-2<sup>k</sup>), BALB/c (H-2<sup>d</sup>), DBA/2 (H-2<sup>d</sup>), and C3H/HeJ (H-2<sup>k</sup>) mice, 6- to 12-wk-old (from Harlan-Sprague-Dawley, Indianapolis, IN, or The Jackson Laboratory, Bar Harbor, ME). In some experiments, T cells were depleted by *in vitro* treatment with anti-Thy-1 antibodies and C or injection of goat antimouse thymocyte serum followed by *in vitro* treatment with monoclonal IgM anti-Thy-1.2 and C (21, 22); these cells were totally unresponsive to PHA and Con A (stimulation indexes  $\leq 1$ ) (21). The following tumor cell lines were obtained from American Type Culture Collection (ATCC), Rockville, MD and cultured as before (23): P815 (H-2<sup>d</sup>, from DBA/2 mice), EL4 (H-2<sup>b</sup>, from C57BL/6 mice), R1.1 (H-2<sup>k</sup>, from C58/J mice), and YAC-1 (H-2<sup>a</sup>, from A/Sn mice).

**MLC.** Primary one-way allogeneic MLC were established by mixing responder lymphocytes (usually from male mice) with mitomycin C-

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<sup>1</sup> This work was supported by United States Public Health Service Grant AR 36640 from National Institutes of Health and a grant from the American Heart Association, Indiana Affiliate.

<sup>2</sup> Abbreviations used in this paper: ECGF, endothelial cell growth factor; FBS, fetal bovine serum.

treated (50 µg/ml, 30 min) stimulator lymphocytes at optimal concentrations ( $10^6$  of each cells/ml, unless otherwise indicated) in RPMI 1640 with 10% FBS (Flow Laboratories, Inc., McLean, VA or HyClone Laboratories, Logan, UT), 50 µM 2-ME, and 1% antibiotic-antimycotic mixture (24), or in some experiments in serum-free HL-1 medium (Ventrex Labs., Portland, ME) with 10 mM HEPES, 2 mM glutamine, 50 µM 2-ME, and 1% antibiotic-antimycotic mixture. For DNA synthesis assays, 0.2-ml cultures were established in triplicate in flat-bottom 96-well plates, and incubated at 37°C in humid 5% CO<sub>2</sub>/95% air for 2 to 7 days (24). For Cr-release cytotoxicity assays, 2.5 ml cultures were established in 24-well plates and incubated for 3 to 7 days (24). To study antitumor responses, mice were primed by an i.v. injection of live histocompatible tumor cells, because cytotoxic cells against histocompatible tumors could not be generated in primary mixed lymphocyte-tumor cultures (the doses, mice, and tumors are indicated in Results). After 16 to 21 days splenic lymphocytes from tumor-primed mice were used as responder cells to establish cultures with mitomycin-treated stimulator tumor cells in RPMI-FBS medium as described above for allogeneic MLC. Experimental groups received different concentrations of various additives specified in Results (heparin, etc.), added at the initiation of the cultures or at other indicated times.

**Measurement of DNA synthesis.** MLC were pulsed with 0.5 µCi [<sup>3</sup>H]TdR for the final 18 h of incubation and the radioactivity incorporated into the cells was determined by the liquid scintillation counting (21, 24). Responder or stimulator cells cultured alone showed negligible background or virtually no [<sup>3</sup>H]TdR incorporation. Mitogenicity was tested in 2-day cultures as before (21). Two-tailed Student's *t*-test was used to analyze the results.

**Cr-release assay.** The effector cells were harvested, washed twice, and suspended in RPMI 1640 with 5% FBS. As target cells, <sup>51</sup>Cr- (Du Pont NEN, Wilmington, DE) labeled (24) appropriate tumor cells or Con A blasts (splenic lymphocytes cultured for 2 to 3 days with Con A and extensively washed with medium containing 25 mM methyl-α-D-mannopyranoside) were used. Target cells ( $10^4$ /well) were mixed with various dilutions of effector cells in 0.2-ml volumes in quadruplicates in round-bottom 96-well plates, to yield the desired E/T ratios. Additional wells with the same numbers of target cells and control unimmunized cells (fresh splenic lymphocytes from the same strain of mice as the responder cells) were also included and showed no or minimal cytotoxicity (within ± 5% at 40/1 E/T ratio). The amount of <sup>51</sup>Cr released after 4 h of incubation was then measured. Spontaneous Cr release was determined from wells containing target cells in the medium alone without the effector cells (it was 5 to 15% of the maximum release for tumor targets and 10 to 20% for Con A blasts), and the maximum release was determined from wells in which target cells were lysed with 0.4% Tween. The results of MLC responses are expressed as % cytotoxicity =  $100 \times ((\text{Cr release with the effector cells}) - (\text{spontaneous Cr release})) / ((\text{maximum Cr release}) - (\text{spontaneous Cr release}))$ . The results are also expressed as LU<sub>20</sub>/per culture or per  $10^7$  recovered viable cells (24), calculated by the regression analysis: LU<sub>20</sub> = number of effector cells needed to lyse 20% of  $10^4$  target cells. Two-tailed Student's *t*-test, paired *t*-test, and Wilcoxon signed rank test (Macintosh StatWorks by Cricket Software, Philadelphia, PA) were used to analyze the results.

**IL-2 assay.** IL-2 was assayed by the [<sup>3</sup>H]TdR incorporation method using the IL-2-dependent CTLL-2 cloned cytotoxic T cells (25) obtained from ATCC and maintained as recommended by ATCC. The IL-2 concentrations (U) were calculated using the computer program (26) kindly provided by Dr. P. Marder from Eli Lilly & Co., Indianapolis, IN.

**Assays for anticoagulant activity.** Anticoagulant activity of heparin and heparinoids was determined by standard activated partial thromboplastin time and thrombin time tests (27) using human plasma.

**Heparinase and protamine treatments.** Heparin digestion with heparinase II (1 U of heparinase/172 µg heparin) was performed in HBSS at 37°C for 18 h, followed by 3 days of dialysis against HBSS at 4°C (12,000 cutoff). Control preparations of heparin were incubated without heparinase and then dialyzed. For protamine treatment, 255 USP U/ml of heparin was incubated with an equivalent concentration (1.6 mg/ml) of protamine at 4°C overnight, the heparin-protamine precipitate was removed by centrifugation in an Eppendorf (Hamburg, West Germany) microfuge, and the supernatant was added to the MLC, to yield the equivalent of 10 USP U/ml of heparin (if it had not been removed by protamine precipitation). As a protamine control, 15 µg/ml of protamine was added to the cultures, which corresponded to the amount of protamine that was left in the above supernatants (determined by the TCA-Lowry assay) after the removal of heparin-protamine precipitate.

**Reagents.** Heparin (160 to 175 USP U/mg from porcine intestinal mucosa) was obtained from Sigma Chemical Co. (St. Louis, MO),

Abbott Laboratories (North Chicago, IL), and Upjohn (Kalamazoo, MI). Low m.w. heparin (m.w. 4000 to 6000), de-N-sulfated heparin, heparan sulfate (from bovine kidney), dermatan sulfate (from porcine skin), chondroitin 4-sulfate (from bovine trachea), chondroitin 6-sulfate (from shark cartilage), dextran sulfate (m.w. 500,000), fucoidan, carrageenan (mostly  $\kappa$ ), polyanetholesulfonate, hyaluronic acid (from human umbilical cord), *Escherichia coli* endotoxin (phenol extract), and other preparations and chemicals were obtained from Sigma, unless otherwise indicated. Recombinant murine IL-2 was from Genzyme Corp. (Boston, MA), purified human monocyte IL-1 from Cistron Biotechnology (Pine Brook, NJ), and purified bovine brain  $\alpha$ -ECGF from Collaborative Research (Bedford, MA). No significant endotoxin contamination of heparin and heparinoids, using the Limulus assay (E-Toxate, Sigma), was detected ( $\leq 0.03$  ng endotoxin/mg of heparin or other compounds). For these tests, the titration of endotoxin standards was done in the presence of 2.5 mg/ml of heparin, because heparin somewhat inhibited the formation of the gel in the Limulus assay.

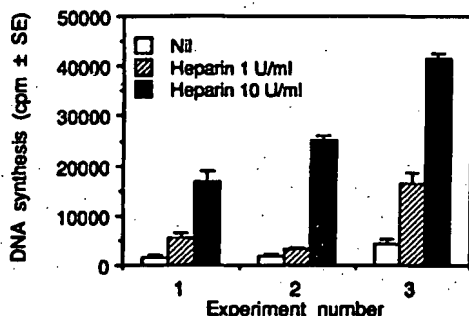
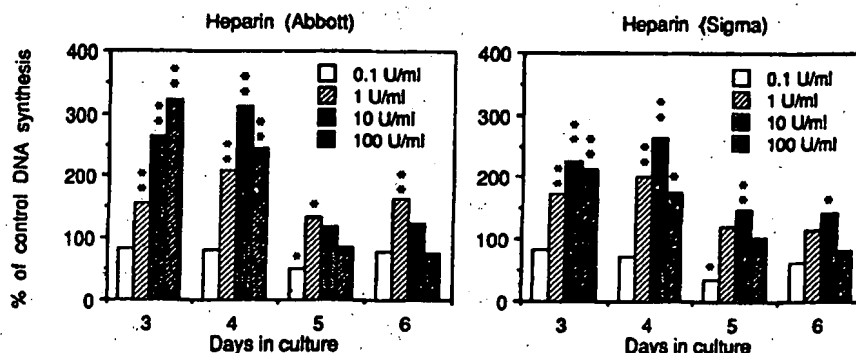
## RESULTS

**Enhancement of allogeneic MLC by heparin.** Heparin enhanced the responses of lymphocytes, when added to the primary allogeneic mouse MLC. This enhancement was demonstrated by increased DNA synthesis in the responding lymphocytes (Figs. 1 and 2) and increased generation of cytotoxic cells (Fig. 3). The maximal enhancement occurred at 1 to 10 USP U/ml and in both assays was evident throughout the entire culture period. At 0.1 USP U/ml, heparin still significantly enhanced generation of cytotoxic cells (see Table III below), but it inhibited DNA synthesis in MLC (Fig. 1). When MLC were established under suboptimal conditions (serum-free medium and lower concentrations of responder and stimulator cells), which did not result in a strong positive allogeneic response in control cultures, much greater enhancement of DNA synthesis by heparin in the responder cells was observed (Fig. 2), as compared with the enhancement of the responses under the optimal culture conditions.

Heparin increased the activity of cytotoxic cells on per cell basis, which resulted in higher cytotoxicity in heparin-treated than in untreated cultures at the same E/T ratios and in increased numbers of LU<sub>20</sub>/10<sup>7</sup> recovered viable cells (Fig. 3). Heparin also increased the yields of viable cells recovered from cultures. Mean  $\pm$  SE ( $\times 10^{-6}$ ) cell yields/culture in MLC were as follows: C57BL/6 anti-CBA/J control (nil),  $1.47 \pm 0.09$ ; heparin (10 USP U/ml),  $2.50 \pm 0.21$  (170% of nil);  $n = 11$ ;  $p < 0.001$  (Student's *t*-test); CBA/J anti-C57BL/6 control (nil),  $1.79 \pm 0.09$ ; heparin (10 USP U/ml),  $2.86 \pm 0.19$  (160% of nil);  $n = 10$ ;  $p < 0.001$ . These increases in cell yields also included cytotoxic cells, because heparin-induced enhancement was appropriately greater when the results were expressed as LU<sub>20</sub>/culture compared to LU<sub>20</sub>/10<sup>7</sup> viable cells (Fig. 3 and other results below).

The heparin-enhanced cytotoxic cells were CTL because: 1) they were T lymphocytes, i.e., the cytotoxic activity was not generated or abolished when the responder cells or effector cells, respectively, were depleted of T cells (not shown); 2) they were cytolytic in a short-time Cr-release assay; 3) their generation required stimulation by allogeneic cells; and 4) they were specific for the H-2 Ag on the stimulator cells (Table I). The cytotoxicity of these CTL and its enhancement by heparin were similar when assayed with the Con A blasts of the stimulator cells or tumor cell lines of appropriate H-2 haplotype, and very little or no cytotoxicity was obtained with tumor targets of the same H-2 haplotype as the responder

**Figure 1.** Effect of heparin on DNA synthesis in MLC. MLC (C57BL/6 anti-CBA/J) were incubated in serum-free HL-1 medium under optimal stimulating conditions (see *Materials and Methods*) without or with the indicated concentrations of heparin, and assayed for DNA synthesis ( $^3\text{H}$ ) TdR incorporation) on the indicated days. The results are derived from 12 to 18 cultures from 4 to 6 experiments; mean control (without heparin) cpm/culture  $\pm$  SE were: day 3, 10,393  $\pm$  1149; day 4, 17,991  $\pm$  2616; day 5, 38,267  $\pm$  3657; day 6, 27,390  $\pm$  3379; \*  $P < 0.02$ ; \*\*  $P < 0.002$  (heparin vs control, *t*-test). Mean cpm/culture  $\pm$  SE of spleen cells from C57BL/6 mice alone (without stimulator cells) were: day 3, 1,006  $\pm$  122; day 4, 242  $\pm$  80; day 5, 179  $\pm$  39, day 6, 175  $\pm$  30.



**Figure 2.** Enhancement of DNA synthesis by heparin in MLC under suboptimal conditions. MLC (C57BL/6 anti-CBA/J) were incubated in serum-free HL-1 medium at half of the optimal cell density ( $0.5 \times 10^6$  of each cells/ml) without or with the indicated concentrations of heparin (Abbott), and assayed for DNA synthesis ( $^3\text{H}$ )TdR incorporation) on day 4. All heparin vs nil differences were statistically significant ( $p < 0.001$ , *t*-test). Mean cpm/culture  $\pm$  SE of spleen cells from C57BL/6 mice alone (without stimulator cells) were: 87  $\pm$  27, 169  $\pm$  29, and 102  $\pm$  13, in experiment 1, 2 and 3, respectively.

cells or with Con A blasts targets of the H-2 haplotype different from the H-2 of the stimulator cells (Table I). The specificity of these CTL was somewhat less restricted against some other tumor targets that were H-2-incompatible with the stimulator and responder cells, however, the sensitivity to lysis of various tumor targets and the enhancement by heparin were not correlated with their sensitivity to NK cells, e.g., YAC-1 (H-2<sup>d</sup>, NK-sensitive) or P815 (H-2<sup>d</sup>, NK-resistant) showed similar degree of sensitivity to CBA/J (H-2<sup>k</sup>) anti-C57BL/6 (H-2<sup>b</sup>) CTL (not shown).

Heparin-induced enhancement of primary allogeneic MLC responses was observed with several responder-stimulator cell combinations, i.e., C57BL/6 anti-CBA/J, CBA/J anti-C57BL/6, and C57BL/6 anti-C3H/HeJ. The enhancement was also observed with various concentra-

tions of the responder and stimulator cells in MLC, ranging from  $0.5$  to  $2 \times 10^6$  of each cells/ml (not shown). The effective enhancement of MLC responses by heparin was observed when heparin was added during the first 24 h of culture (Fig. 4). Heparin was much less effective or not effective when added at 48 or 72 h of culture (Fig. 4).

The enhancement of allogeneic MLC responses was due to heparin itself and not to any possible contaminants that might be found in heparin preparations, because: 1) similar enhancement was observed with four different batches of heparin from three different manufacturers; 2) heparin preparations were not contaminated with endotoxin (see *Materials and Methods*) and endotoxin at  $\leq 1$  ng/ml (i.e., at 10,000 times higher concentration than the possible endotoxin contamination from heparin used at 1 USP U/ml) did not enhance the generation of cytotoxic cells (not shown); 3) the enhancing effect of heparin was inhibited by protamine (Fig. 5), which binds to heparin and acts as a heparin antagonist in other systems (1, 2, 28, 29); 4) the enhancing effect of heparin was greatly diminished by digestion with heparinase and subsequent dialysis, whereas the dialysis by itself did not diminish or even increased the enhancing activity of heparin (Fig. 5); and 5) there was no enhancement of MLC responses by similar concentrations of some heparinoids (see below) or unrelated substances, e.g., gelatin (not shown).

The effect of heparin was not due to a simple carry-over of heparin from the cultures into the Cr-release assay because: 1) the effector cells were extensively washed before the assay; 2) there was very little or no effect of heparin, when it was added during the last 2 days of culture (Fig. 4); and 3) the addition of heparin directly to the Cr-release assay inhibited (rather than enhanced) the lysis of target cells in a concentration-dependent manner (inhibition by 36% at 1 USP U/ml,

**Figure 3.** Enhancement of generation of CBA/J anti-C57BL/6 cytotoxic cells in MLC by heparin. MLC were incubated in RPMI + 10% FBS medium alone or with 1 or 10 U/ml of heparin, and assayed for cytotoxicity on the indicated days by the Cr-release assay with EL4 targets at 2/1, 10/1, and 40/1 E/T ratios; LU<sub>50</sub> were calculated based on percent cytotoxicity as defined in *Materials and Methods*. The results are based on means of four cultures from one representative experiment of four similar experiments. All cytotoxicity values (except on day 3) were significantly higher (by *t*-test) in heparin-treated than in control (nil) cultures.

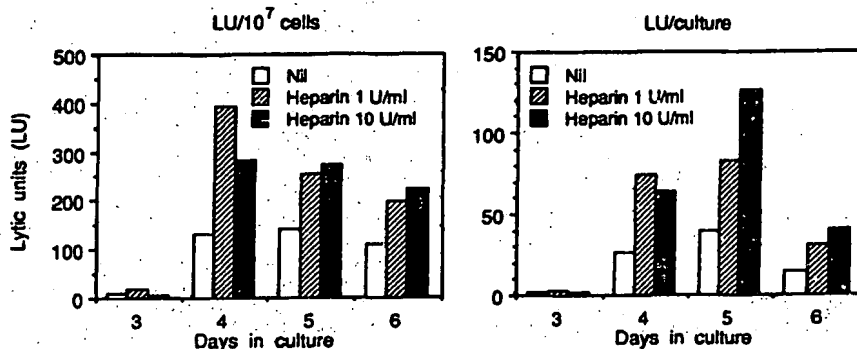


TABLE I  
Specificity of heparin-enhanced CBA/J (H-2<sup>a</sup>) anti-C57BL/6 H-2<sup>a</sup> cytotoxic cells<sup>a</sup>

Additions to Cultures and E/T Ratios	% Cytotoxicity (LU <sub>50</sub> /Culture) with following Target Cells							
	C57BL/6 Con A blasts (H-2 <sup>b</sup> )		EL4 (H-2 <sup>b</sup> )		R1.1 (H-2 <sup>b</sup> )		BALB/c Con A blasts (H-2 <sup>b</sup> )	
Nil								
2/1	0.4 ± 0.3		3.8 ± 0.2		0.3 ± 0.4		-2.3 ± 0.6	
10/1	5.1 ± 0.7	(14.8)	12.4 ± 0.9	(19.7)	2.8 ± 0.2	(0.0001)	-0.8 ± 0.4	(0.0005)
40/1	40.2 ± 1.0		42.9 ± 1.8		4.7 ± 0.3		3.0 ± 0.5	
Heparin (Sigma) (10 U/ml)								
2/1	8.1 ± 1.7		12.0 ± 1.0		0.4 ± 0.2		-1.8 ± 0.9	
10/1	35.1 ± 2.5	(65.1)	34.4 ± 2.1	(73.2)	2.6 ± 0.3	(0.002)	1.8 ± 0.8	(0.16)
40/1	65.2 ± 2.4		64.8 ± 1.5		5.8 ± 0.02		8.0 ± 1.2	
Heparin (Upjohn) (10 U/ml)								
2/1	5.8 ± 0.4		10.1 ± 0.8		0.3 ± 0.1		-0.7 ± 0.4	
10/1	29.0 ± 0.5	(42.5)	31.5 ± 1.4	(51.6)	1.8 ± 0.2	(0.0009)	-0.1 ± 0.2	(0.12)
40/1	59.2 ± 0.8		61.2 ± 1.9		5.2 ± 0.6		7.4 ± 0.7	

<sup>a</sup> MLC (CBA/J anti-C57BL/6) were incubated for 4 days in RPMI + 10% FBS medium alone or with heparin, and assayed with the indicated <sup>51</sup>Cr-labeled target cells. The results are means ± SE derived from eight cultures from two experiments.

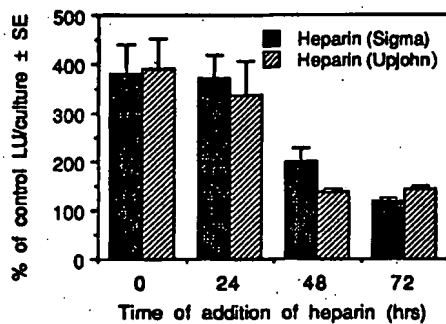


Figure 4. Effect of delayed addition of heparin on the generation of cytotoxic cells in MLC. MLC (CBA/J anti-C57BL/6) were incubated for 4 days in RPMI + 10% FBS medium alone or with 10 U/ml of heparin added at the indicated time, and assayed with <sup>51</sup>Cr-labeled EL4 target cells. The results are mean percent of control from three experiments. In control cultures (without heparin) mean ± SE LU<sub>50</sub>/culture was 16.3 ± 3.1. Similar results were obtained when the data were expressed as LU<sub>50</sub>/10<sup>7</sup> viable cells (not shown).

53% at 10 USP U/ml, and 99% at 100 USP U/ml; means from three experiments). This inhibition was not due to the binding and depletion of Ca<sup>2+</sup> by heparin, because the addition of more Ca<sup>2+</sup> to the cytotoxicity assay did not overcome this inhibition (not shown). Another simplistic explanation of heparin-induced enhancement, i.e., that heparin inhibited the cytolytic activity of CTL during the culture, thus preventing "exhaustion" of the cytolytic factors (which then could be used more effectively during the Cr-release assay) also seems unlikely, because hepa-

rin was only very weakly effective or not effective when added at 48 or 72 h of culture (Fig. 4). Heparin was also not mitogenic for mouse lymphocytes in a standard [<sup>3</sup>H] TdR incorporation assay after 2 days of culture (see Table III below); somewhat higher background [<sup>3</sup>H]TdR incorporation on days 4 to 6 in some cultures containing heparin and responder cells alone (≤1000 cpm/culture) were most likely due to improved viability of the cells.

**Enhancement of antitumor cytotoxic responses by heparin.** Heparin at 0.1 to 10 USP U/ml also enhanced generation of cytotoxic responses against H-2 histocompatible tumors when present in the mixed lymphocyte-tumor cultures of lymphocytes from BALB/c mice primed with 100 P815 cells (Table II). Significant enhancements of antitumor responses were also obtained with lymphocytes from BALB/c mice primed with higher doses of P815 cells (10<sup>3</sup>, 10<sup>4</sup>, and 10<sup>5</sup> cells/mouse; acute 30-day i.v. LD<sub>50</sub> of P815 tumor for BALB/c mice was 10<sup>5.25</sup> cells) (not shown). The cytotoxic cells were specific to the tumor Ag because of total lack of cytotoxicity of BALB/c anti-P815 cells against DBA/2 blast targets (not shown). Heparin also enhanced cytotoxic responses of primed CBA/J mice against an H-2 histocompatible R1.1 tumor (not shown). Antitumor responses were different from antiallogeneic cell responses in four respects: 1) they required *in vivo* priming with tumor cells; 2) they usually peaked later (on day 6); 3) they could be more effectively enhanced with lower concentration of heparin; and 4) heparin enhanced the cytotoxicity without significantly in-

Figure 5. Inhibition of heparin-induced enhancement of cytotoxic responses by protamine or by digestion of heparin with heparinase. MLC (CBA/J anti-C57BL/6) were incubated for 4 days in RPMI + 10% FBS medium alone or with 10 U/ml of heparin, dialyzed heparin, heparinase-digested and dialyzed heparin, supernatant from protamine-precipitated heparin, or protamine control (see Materials and Methods for details), added at the initiation of the cultures. The results are based on LU<sub>50</sub>/culture calculated from mean cytotoxicity values assayed with <sup>51</sup>Cr-labeled EL4 target cells in two representative experiments. In control cultures (without heparin or other additives) LU<sub>50</sub>/culture were 7.6 and 11.3 in experiment 1 and 2, respectively. Similar results were obtained when the data were expressed as LU<sub>50</sub>/10<sup>7</sup> viable cells (not shown).

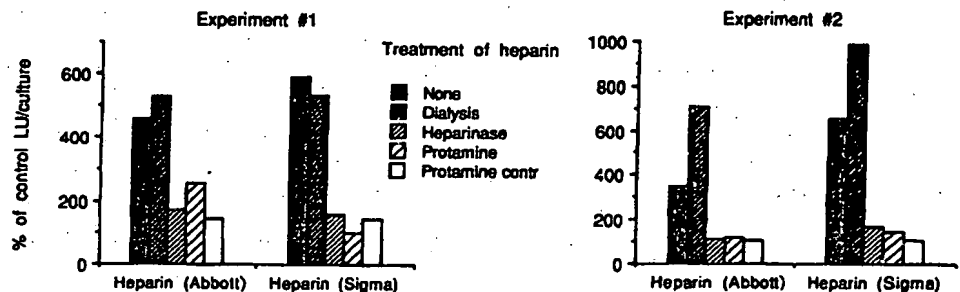


TABLE II  
Enhancement of generation of BALB/c anti-P815 cytotoxic cells in mixed lymphocyte-tumor cultures by heparin\*

Additions to Cultures and E/T Ratios	Day 4	Day 5 (% cytotoxicity (LU <sub>50</sub> /culture))	Day 6
<b>Nil</b>			
2/1	1.1 ± 0.3	5.0 ± 0.3	4.4 ± 0.3
10/1	9.7 ± 0.3 (8.5)	18.2 ± 0.8 (17.4)	19.1 ± 0.6 (19.5)
40/1	32.7 ± 0.4	45.3 ± 0.5	54.6 ± 2.5
<b>Heparin (0.1 U/ml)</b>			
2/1	4.0 ± 0.5	6.5 ± 0.1	10.2 ± 0.7
10/1	21.0 ± 0.6 (20.5)	22.9 ± 1.4 (17.1)	41.6 ± 2.3 (34.4)
40/1	55.6 ± 0.6	47.5 ± 1.4	68.6 ± 3.5
<b>Heparin (1 U/ml)</b>			
2/1	5.3 ± 0.6	11.7 ± 0.2	13.3 ± 0.9
10/1	21.3 ± 1.0 (21.2)	42.9 ± 0.2 (37.3)	47.4 ± 0.5 (47.7)
40/1	57.0 ± 3.1	66.6 ± 2.6	80.1 ± 1.2
<b>Heparin (10 U/ml)</b>			
2/1	2.7 ± 0.4	7.2 ± 0.1	11.4 ± 0.3
10/1	12.3 ± 0.8 (14.1)	27.0 ± 0.7 (30.9)	46.4 ± 1.9 (47.3)
40/1	39.5 ± 3.3	61.6 ± 2.6	75.5 ± 1.6

\* BALB/c (H-2<sup>d</sup>) mice were primed with 100 P815 (H-2<sup>k</sup>) cells. After 16 days, their spleen cells were cultured with mitomycin-treated P815 cells in RPMI + 10% FBS medium alone or with heparin, and assayed on the indicated days by the Cr-release assay with P815 targets. The results are means ± SE of four cultures from one representative experiment of three similar experiments. Most cytotoxicity values were significantly higher at  $p < 0.001$  (by *t*-test) in heparin-treated than in control (nil) cultures.

creasing the total numbers of viable cells in cultures.

**Effect of various heparinoids on generation of cytotoxic cells in MLC and correlation with their mitogenic and anticoagulant activities.** To study the structure-function relationships of the enhancement of cytotoxic responses, we next compared the anticoagulant, mitogenic, and MLR-enhancing activities of heparins and various natural (animal and plant) and synthetic heparinoids (Table III). Heparin was the best enhancer of cytotoxic responses. Low m.w. heparin was also active, but required higher concentrations. De-N-sulfation reduced the enhancing activity of heparin more than 10 times. It is not clear if the remaining activity was due to incomplete de-N-sulfation, which is 94 to 98.5% effective (according to Sigma) and reduces the anticoagulant activity >800 times, or if it is due to some other structural features of heparin. None of the other sulfated heparinoids (regardless whether more or less sulfated than heparin) significantly enhanced the cytotoxic response, whereas nonsulfated hyaluronic acid was active, but less than heparin and at 10 times higher concentration. Some of the heparinoids (dextran sulfate, fucoidan, polyanetholesulfonate, and hyaluronic acid) greatly inhibited generation of cytotoxic cells. This inhibitory effect was not due to nonspecific toxicity of these compounds, because even at concentrations that totally inhibited cytotoxic responses they did not reduce cell viability in cultures, and even some of them significantly increased the numbers of viable cells recovered from the cultures (fucoidan and polyanetholesulfonate, 183 and 187% of control, respectively, at 65 µg/ml). Therefore, there was no correlation between the extent of sulfation of heparinoids and the enhancement or inhibition of cytotoxic responses, indicating that the presence of a negative charge or sulfate groups by themselves were not sufficient for these immunomodulating effects. The positions of the sulfate groups on these molecules, however, may be important, because de-N-sulfation of heparin markedly reduced its immunoenhancing activity.

Although heparin and low m.w. heparin were the most effective anticoagulants and immunoenhancers, there

was no correlation between anticoagulant and immunomodulating activities among other heparinoids; e.g., dextran sulfate, fucoidan, and polyanetholesulfonate had a good anticoagulant activity, but did not enhance and even suppressed cytotoxic responses, whereas de-N-sulfated heparin and hyaluronic acid had very weak or no anticoagulant activity, but significantly enhanced cytotoxic responses (Table III). There was also no correlation between the mitogenic activity of heparinoids and their immunomodulating or anticoagulant activities. The mitogenic activity also did not correlate with the extent of sulfation (Table III).

**Effect of heparin on activity of IL-2, IL-1, and ECGF.** Because heparin was recently shown to enhance production of IL-2 by PHA- and PWM-stimulated human mononuclear cells (20) and because IL-2 is the main lymphokine needed for the generation of CTL (25, 30), we evaluated the effect of heparin on the generation of IL-2 in MLC. Heparin accelerated production of IL-2 in MLC (Table IV), however, heparin did not enhance IL-2 production, because the peak concentration of IL-2 in heparin-containing cultures was not higher than in the control cultures, and the total amounts of IL-2 released into the medium throughout 5 days of incubation (assayed daily) were the same in heparin-containing and control cultures, despite marked enhancement of cytotoxic activity in the same cultures throughout the entire incubation period. These results should be interpreted with caution, because assaying IL-2 in culture supernatants does not totally account for rapid consumption of secreted IL-2 by the growing cells. However, as already indicated, enhancement of cytotoxic activity by heparin greatly exceeded the increases in cell numbers in MLC.

Heparin neither enhanced nor interfered with the growth-promoting activity of soluble IL-2 on mouse T lymphocytes, because the addition of heparin together with IL-2 to the cultures of CTLL-2 cloned cytotoxic T cells for the entire duration of the IL-2 assay did not significantly change the response of these cells to IL-2 (e.g., determination of IL-2 concentration in a rat spleen cell Con A supernatant in the absence of heparin or in



TABLE III

Comparison of effects of various heparinoids on generation of cytotoxic cells in MLC with their anticoagulant and mitogenic activities

Heparin(oid)	Sulfation <sup>a</sup> (mol sulfate/ disaccharide)	Anticoagulant activity <sup>b</sup>		Concentration ( $\mu$ g/ml)	Mitogenicity <sup>c</sup> Stimulation index	Effect on cytotoxicity <sup>d</sup> (% of control LU <sub>50</sub> /culture)
		APTT ( $\mu$ g/ml)	TT ( $\mu$ g/ml)			
Heparin	2-3	0.27	0.11	0.62 <sup>e</sup> 6.2 62 620	1.0 0.9 0.8 1.0	155 $\pm$ 14/ 296 $\pm$ 41/ 385 $\pm$ 46/ 109 $\pm$ 10
Low m.w. heparin	2-3	1.4	0.28	0.62 6.2 62 620	1.0 1.1 1.0 1.3	104 $\pm$ 29 177 $\pm$ 29/ 244 $\pm$ 26/ 413 $\pm$ 35/
DE-N-sulfated heparin	1-2	221	95	0.62 6.2 62	1.1 1.0 1.2	101 $\pm$ 21 149 $\pm$ 55 225 $\pm$ 65/
Heparan sulfate	1-2	45	17	6.5 65	1.3 2.4	134 $\pm$ 22 153 $\pm$ 28
Dermatan sulfate	1	76	47	6.5 65	0.9 1.1	115 $\pm$ 13 122 $\pm$ 23
Chondroitin 4-sulfate	1	398	188	6.5 65	1.1 1.8	108 $\pm$ 8 96 $\pm$ 6
Chondroitin 6-sulfate	1	>2500	>2500	6.5 65 650	0.7 0.7 4.3	101 $\pm$ 15 79 $\pm$ 12 NT
Dextran sulfate	4	2.9	1.0	6.5 65	1.5 0.8	119 $\pm$ 29 0 $\pm$ 0/
Fucoidan	2	11	4.3	6.5 65 650	4.5 14.0 41.1	163 $\pm$ 54 2 $\pm$ 0.5/ NT
Carrageenan	1	85	32	6.5 65 650	0.8 1.2 4.7	89 $\pm$ 34 84 $\pm$ 29 NT
Polyanetholesulfonate	2	12	8.5	6.5 65	1.5 1.0	60 $\pm$ 14/ 20 $\pm$ 15/
Hyaluronic acid	0	>2500	>2500	6.5 65 650	1.3 4.9 22.4	93 $\pm$ 12 238 $\pm$ 18/ 0 $\pm$ 0/

<sup>a</sup> Approximate values from References 2, 3, 40.

<sup>b</sup> Concentration that doubles APTT or TT.

<sup>c</sup> CBA/J spleen cells were incubated for 2 days in RPMI + 5% FBS medium alone or with the indicated products added at the initiation of the cultures. DNA synthesis was measured by incorporation of [<sup>3</sup>H]TdR; the stimulation index = [<sup>3</sup>H]TdR incorporation in cultures with the products/thymidine incorporation in cultures in medium alone (which was 1004  $\pm$  22, mean cpm  $\pm$  SE); the results are means of two experiments.

<sup>d</sup> MLC (CBA/J anti-C57BL/6) in RPMI + 10% FBS medium alone (control) or with the indicated products added at the initiation of the cultures were assayed after the optimal incubation period (4 to 5 days) for cytotoxic cells with <sup>51</sup>Cr-labeled EL4 targets. The results are mean percent of control LU<sub>50</sub>/culture  $\pm$  SE from four to six experiments; mean  $\pm$  SE control LU<sub>50</sub>/culture was 46.2  $\pm$  10.6; NT, not tested.

<sup>e</sup> 0.62  $\mu$ g/ml heparin equals 0.1 USP U/ml.

<sup>f</sup> LU<sub>50</sub> significantly different ( $p \leq 0.05$ ) from control by paired *t*-test.

TABLE IV

 Comparison of effect of heparin on IL-2 concentration and generation of cytotoxic cells in MLC<sup>a</sup>

Additions to Cultures	IL-2 Concentrations (IL-2 U/ml (% of Nil))					
	Day 1	Day 2	Day 3	Day 4	Day 5	Total IL-2 (day 1 + 2 + 3 + 4 $\pm$ 5)
Nil	0.22	0.95	1.94	0.79	0.13	4.04
Heparin (1 U/ml)	0.37 (168)	1.40 (147)	1.73 (89)	0.42 (53)	0.17 (128)	4.09 (101)
Heparin (10 U/ml)	0.37 (167)	1.20 (126)	1.66 (86)	0.52 (65)	0.25 (185)	4.00 (99)
	Cytotoxic Cells (LU <sub>50</sub> /Culture (% of Nil))					
	Day 3	Day 4	Day 5	Day 6		
Nil	0.9	11.5	18.0	11.7		
Heparin (1 U/ml)	1.5 (168)	45.7 (397)	59.0 (328)	27.3 (233)		
Heparin (10 U/ml)	1.1 (127)	36.8 (320)	72.8 (404)	27.6 (236)		

<sup>a</sup> MLC were incubated and assayed for cytotoxicity as described in Figure 3. Aliquots of supernatants were also assayed for the presence of IL-2 using CTLL-2 cells as described in *Materials and Methods*. The results are means of two experiments.

the presence of 0.1, 1, and 10 USP U of heparin/ml, respectively, yielded 12.7, 12.8, 11.8, and 12.2 IL-2 U/ml).

Because heparin was shown to synergistically enhance the growth-promoting effects of several growth factors (mainly endothelial growth factors) (4, 5, 28, 31-33), we looked for possible synergistic effects of heparin and lymphokines (lymphocyte growth factors). Because for its enhancing effect heparin had to be present during early stages of MLC (first 24 h), we selected two major lympho-

kines (IL-1 and IL-2) that are induced early in the immune response. In addition, we used acidic ECGF, whose action on the cells of mesodermal origin is greatly enhanced by heparin (4, 5, 28, 31, 33), but whose effects on the immune system are largely unknown. Costimulation of MLC with heparin and IL-2 did not result in enhanced generation of cytotoxic cells, as compared with the effects of heparin alone or IL-2 alone (Table V), as reflected by the similar values of the actual enhancement obtained in costimulated cultures and the calculated expected en-



TABLE V  
Costimulation of MLC with heparin and IL-2<sup>a</sup>

Additions to Cultures	Day 4		Day 5		Day 6	
	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture	LU <sub>50</sub> /10 <sup>7</sup> cells (% of control without heparin and IL-2)	LU <sub>50</sub> /culture	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture
Heparin (1 U/ml)	335 ± 91	331 ± 65	199 ± 28	286 ± 47	193 ± 25	207 ± 19
Heparin (10 U/ml)	238 ± 50	319 ± 54	208 ± 21	383 ± 55	199 ± 37	285 ± 41
IL-2	124 ± 13	133 ± 11	118 ± 24	132 ± 28	226 ± 54	191 ± 41
IL-2 + heparin (1 U/ml)	298 ± 71	415 ± 99	210 ± 31	263 ± 45	230 ± 42	236 ± 30
(expected % of control) <sup>b</sup>	(416)	(440)	(235)	(378)	(437)	(395)
IL-2 + heparin (10 U/ml)	322 ± 98	495 ± 92	261 ± 53	539 ± 65	202 ± 31 <sup>c</sup>	269 ± 35 <sup>c</sup>
(expected % of control) <sup>b</sup>	(296)	(425)	(246)	(506)	(450)	(543)

<sup>a</sup> MLC (CBA/J anti-C57BL/6) were incubated in RPMI + 10% FBS medium alone (control), with heparin alone, IL-2 alone (25 NIH U/ml assayed by Genzyme Corp., which corresponded to 2.5 U/ml in our assay), or heparin and IL-2. After 4, 5, or 6 days, the cultures were assayed for cytotoxic cells with <sup>51</sup>Cr-labeled EL4 cells. The results are mean percent of control ± SE from six to eight experiments. The mean control values ± SE (LU<sub>50</sub>/10<sup>7</sup> cells and LU<sub>50</sub>/culture, respectively) were as follows: day 4, 176 ± 38 and 40.3 ± 10.8; day 5, 194 ± 26 and 46.2 ± 10; and day 6, 91 ± 8 and 14.7 ± 2.9.

<sup>b</sup> Expected percent of control = (% of control for heparin alone) × (% of control for IL-2 alone)/100.

<sup>c</sup> *p* < 0.05 vs expected percent of control by Wilcoxon signed rank test; all other differences of actual vs expected values were not significant (*p* > 0.05).

hancement, which assumes independent effects of the two stimulants. On the last day of culture (day 6), even some interference between the two stimulants was observed, as the actual enhancement was lower than the expected values. This may reflect competition between the effects of heparin and IL-2 on the same cells, because the greatest enhancement by heparin alone was seen on days 4 and 5, whereas IL-2 alone enhanced generation of cytotoxic cells late in the culture (on day 6), most likely because it prolonged the period of cell proliferation. Similar results were obtained when IL-2 was added after 24 h of culture or when five times lower or higher IL-2 concentrations were used (not shown).

Costimulation of MLC with heparin and IL-1 resulted in enhanced generation of cytotoxic cells on the day of maximal response (day 5), as compared with the effects of heparin alone or IL-1 alone (Table VI), as reflected by higher values of the actual enhancement obtained in costimulated cultures than the calculated expected enhancement. Costimulation of MLC with heparin and ECGF did not result in enhanced generation of cytotoxic cells, as compared with the effects of heparin alone (Table VII). ECGF by itself suppressed generation of CTL, which was most pronounced on day 6, and heparin abolished this suppression.

#### DISCUSSION

The results presented here demonstrate that heparin enhances proliferation of cells and generation of CTL in

primary allogeneic MLR in mice. Heparin also enhances cytotoxic responses against histocompatible tumors in mice. The enhancing effect seems to be specific for heparin, since it is only obtained with high and low m.w. heparins, but not with a number of other sulfated heparinoids. Interestingly, some of these heparinoids (including dextran sulfate) totally inhibited generation of cytotoxic cells. Our results show no correlation between the immunomodulating activities of heparinoids and their anticoagulant and mitogenic activities or their extent of sulfation.

There was also no correlation of the immunoenhancing activity of heparinoids with the reported (34, 35) presence of binding sites for sulfated polysaccharides on mouse lymphocytes, because T cells, B cells, and thymocytes (but not other nonlymphoid cells) all have binding sites for heparin, dextran sulfate, fucoidan, and carrageenan, and do not have binding sites for chondroitin-4- and 6-sulfates, dermatan sulfate, heparan sulfate, and hyaluronic acid. The cross-inhibition of binding analysis revealed the presence of at least four different specificities of these binding sites for the individual polysaccharides (34). The biochemical nature and function(s) of these binding sites are not known. It is intriguing, however, that binding of sulfated polysaccharides to the CD2 molecule has been recently reported (36). At least one documented function of the binding sites for sulfated polysaccharides has been their participation in lymphocyte re-

TABLE VI  
Costimulation of MLC with heparin and IL-1<sup>a</sup>

Additions to Cultures	Day 4		Day 5	
	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture (% of control without heparin and IL-1)	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture
Heparin (1 U/ml)	259 ± 32	311 ± 36	207 ± 28	238 ± 16
Heparin (10 U/ml)	295 ± 44	424 ± 56	273 ± 46	356 ± 44
IL-1	130 ± 20	136 ± 28	98 ± 11	105 ± 16
IL-1 + heparin (1 U/ml)	326 ± 48	443 ± 57	315 ± 88	347 ± 80
(expected % of control) <sup>b</sup>	(337)	(423)	(198)	(251)
IL-1 + heparin (10 U/ml)	315 ± 51	429 ± 75	605 ± 151 <sup>c</sup>	669 ± 124 <sup>c</sup>
(expected % of control) <sup>b</sup>	(384)	(576)	(261)	(374)

<sup>a</sup> MLC (CBA/J anti-C57BL/6) were incubated in RPMI + 10% FBS medium alone (control), with heparin alone, IL-1 (1 U/ml) alone, or heparin and IL-1. After 4 or 5 days, the cultures were assayed for cytotoxic cells with <sup>51</sup>Cr-labeled EL4 cells. The results are mean percent of control ± SE from six experiments. The mean control values ± SE (LU<sub>50</sub>/10<sup>7</sup> cells and LU<sub>50</sub>/culture, respectively) were as follows: day 4, 132 ± 38 and 33.0 ± 11.1; day 5, 268 ± 24 and 90.5 ± 12.

<sup>b</sup> Expected percent of control = (% of control for heparin alone) × (% of control for IL-1 alone)/100.

<sup>c</sup> *p* < 0.014 vs expected percent of control by Wilcoxon signed rank test; all other differences of actual vs expected values were not significant (*p* > 0.05).

TABLE VII  
Costimulation of MLC with heparin and ECGF<sup>a</sup>

Additions to Cultures	Day 4		Day 5		Day 6	
	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture	LU <sub>50</sub> /10 <sup>7</sup> cells	LU <sub>50</sub> /culture
	(% of control without heparin and ECGF)					
Heparin (1 U/ml)	313 ± 85	328 ± 77	213 ± 34	325 ± 62	182 ± 26	198 ± 31
Heparin (10 U/ml)	241 ± 63	298 ± 55	226 ± 20	446 ± 66	185 ± 33	280 ± 43
ECGF	80 ± 17	79 ± 16	98 ± 11	109 ± 14	38 ± 14	38 ± 14
ECGF + heparin (1 U/ml)	255 ± 74	335 ± 84	195 ± 17	241 ± 30	139 ± 17	169 ± 12 <sup>c</sup>
(expected % of control) <sup>b</sup>	(251)	(258)	(208)	(354)	(69)	(74)
ECGF + heparin (10 U/ml)	176 ± 20	286 ± 76	204 ± 13	330 ± 24	173 ± 22 <sup>c</sup>	282 ± 35 <sup>c</sup>
(expected % of control) <sup>b</sup>	(193)	(234)	(221)	(486)	(70)	(105)

<sup>a</sup> MLC (CBA/J anti-C57BL/6) were incubated in RPMI + 10% FBS medium alone (control), with heparin alone, ECGF (5 ng/ml) alone, or heparin and ECGF. After 4, 5, or 6 days, the cultures were assayed for cytotoxic cells with <sup>51</sup>Cr-labeled EL4 cells. The results are mean percent of control ± SE from six experiments. The mean control values ± SE (LU<sub>50</sub>/10<sup>7</sup> cells and LU<sub>50</sub>/culture, respectively) were as follows: day 4, 175 ± 44 and 38.1 ± 10.9; day 5, 206 ± 32 and 51.3 ± 11.4; and day 6, 160 ± 63 and 24.9 ± 9.9.

<sup>b</sup> Expected percent of control = (% of control for heparin alone) × (% of control for ECGF alone)/100.

<sup>c</sup> *p* < 0.05 vs expected percent of control by Wilcoxon Signed Rank test; all other differences of actual vs expected values were not significant (*p* > 0.05).

circulation and binding to other cells and to extracellular matrix. Some sulfated polysaccharides (dextran sulfate, heparin, fucoidan) cause profound lymphocytosis in animals, change lymphocyte homing patterns, and inhibit lymphocyte binding to the high endothelial venules (37–40). These characteristics also did not correlate with the adjuvant and anticoagulant properties of these heparinoids (38). The exact distribution of the binding sites for different heparinoids on different lymphocyte subpopulations, however, is not known, and, therefore, a correlation of the immunomodulating activities of these heparinoids with the presence of specific binding site subtypes may still be found.

The effective immunoenhancing concentrations of heparin (0.1 to 10 USP U/ml) are clinically relevant, since they are attained in patients' plasma and persist for at least 8 h (initial 3 USP U/ml, with 1.5 h t<sub>1/2</sub>) after a standard i.v. dose of 10,000 USP U (29). As mentioned above, heparin also binds to lymphocytes and other cells, and it is possible that heparin could continue to exert its immunomodulating effects in a surface-bound form, as is the case for the enhancement of growth factors' effects (6).

Our results showing immunoenhancing effects of heparin extend recent observations of Eskinazi et al. (20) and contradict previous reports of inhibitory effects of heparin on various types of immune responses, mentioned in the Introduction (15–19). Most of these inhibitory effects were seen at higher concentrations of heparin or in different systems and, therefore, direct comparison of these results is difficult. It is well known that most immunostimulants can also act as immunosuppressants under certain conditions. In our system heparin suppressed cytotoxicity when present directly in the cytotoxicity assay, which confirms previous observations and may be due to the interaction of heparin with perforin (41). Another immunosuppressive effect of heparin, i.e., inhibition of DNA synthesis in MLC at low concentrations (0.1 USP U/ml), might have been due to the induction of early cell differentiation, because the same dose of heparin still enhanced the cytotoxic responses. In general, lower concentrations of heparin tended to induce early enhancement of cytotoxicity in cultures, whereas high doses of heparin (100 USP U/ml) induced initial greater enhancement of DNA synthesis with suppression of cytotoxicity on day 3 and 4, and then a delay of the peak of

cytotoxic response until day 6 (data not shown). This delayed peak for high m.w. heparin was usually not higher than the peak of control response (on day 4 or 5). With the same concentration of low m.w. heparin, there was no suppression of cytotoxicity early in the culture and the delayed peak was much higher than the control response.

Some heparinoids (e.g., dextran sulfate) at low nontoxic concentrations (65 µg/ml) completely suppressed generation of cytotoxic cells throughout the entire incubation period (7 days). Inasmuch as dextran sulfate is currently undergoing clinical trials in AIDS patients (11–14), the possibility of suppression of the immune response by the clinically used preparations of dextran sulfate (which are of lower m.w. than the preparation used in this study because of their lower anticoagulant activity) should be thoroughly evaluated.

Although not rigorously excluded, heparin did not seem to have a lectin-like polyclonal-activating effect, or phorbol ester-like or IL-2-like ability to induce promiscuous killing or activation of nonspecific lymphokine-activated killer or NK-like cells, because: 1) the cytotoxic cells were CTL specific for the H-2 Ag of the stimulator cells and, in contrast to lymphokine-activated killer cells (42, 43) did not kill autologous targets and did not show enhanced killing of NK-sensitive targets; 2) their generation required prolonged incubation with the immunizing cells and was not seen in cultures without the stimulator cells; and 3) the enhancement was not seen when heparin was added late during the culture or directly into the cytotoxicity assay, in contrast to phorbol esters which can induce promiscuous killing almost instantaneously and inhibit lytic activity upon prolonged exposure in culture (44). Heparin also did not exert its immunoenhancing effect by inducing higher amounts of IL-2, although some acceleration of IL-2 production was seen in heparin-containing cultures.

Heparin binds to a number of polypeptide growth factors and synergistically enhances their growth-promoting activities, mainly by increasing their affinity for the receptors and also by protecting them from proteolytic degradation (4, 5, 28, 31–33), and possibly by concentrating them in a surface-bound form (6). Therefore, we tested if heparin could synergistically enhance the effects of lymphokines (IL-2 and IL-1) in MLC. Heparin did not enhance the effect of IL-2 in MLC and it had no effect on

the growth-promoting activity of IL-2 on cloned cytotoxic T cells. However, heparin had a synergistic enhancing effect with IL-1 on the generation of cytotoxic cells in MLC. This finding is interesting in view of the fact that IL-1 shares a significant amino acid homology with heparin-binding growth factors (45). Future studies should address questions concerning the mechanism of this enhancement, e.g., whether heparin actually binds to IL-1 and if it enhances other biologic activities of IL-1. We also found out that ECGF suppresses generation of cytotoxic cells in MLC and that heparin can abolish this suppression and still exert its immunoenhancing effect. The mechanism and the target cells of this ECGF-induced immunosuppression are not known. These interactions of heparin with IL-1 and ECGF, however, cannot fully explain the immunoenhancing effect of heparin, because they were only evident late in the culture (day 5 and 6), whereas the enhancement of cytotoxic responses was seen throughout the entire incubation period (i.e., also on day 3 and 4). These results, therefore, suggest that heparin may have multiple immunoenhancing effects, which is not surprising in view of the fact that other biologic effects of heparin, e.g., anticoagulant activity, are also due to multiple mechanisms of action (1, 2). Moreover, the antitumor effect of heparin may be also due to multiple effects, because in addition to inhibition of tumor vascularization (8) and enhancement of tumor cell clearance (9) and attachment (10) in vivo, our studies show that heparin can enhance generation of tumor-specific cytotoxic cells in vitro.

There are multiple potential implications in our findings. The enhancement of cytotoxic responses by heparin may be beneficial in increasing host responses against viral infections and tumors, which may be of special value in AIDS patients, because heparin also inhibits infection of lymphocytes by the HIV virus (11-14). It may also be useful in in vitro attempts to generate tumor-specific cytotoxic cells from tumor-infiltrating lymphocytes (43). However, if the enhancement of allogeneic MLR is a true reflection of the ability to reject grafts, this phenomenon could be potentially very harmful in transplant patients, who receive heparin for prevention or treatment of thrombosis. This underscores the need for full understanding of immunomodulating properties of heparin.

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